Utilization of Chlorella vulgaris for biomass production and treatment of wastewater from greenhouse farms

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Utilization of *Chlorella Vulgaris* for Biomass Production and Treatment of Wastewater From Greenhouse Farms

By

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

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ABSTRACT

In the current research work, green microalgae *C. vulgaris* was used for treating hydroponic and aquaponic wastewater collected from the Center for Applied Research on the Environment and Sustainability (CARES) at The American University in Cairo (New Cairo, Egypt) and for producing biomass. The present study was undertaken to evaluate the use of microalgae for bioremediating wastewater from greenhouse farm and for producing biomass under different conditions and to explore the economic implications of microalgal biofuels, focusing on the effect of different cultivation modes. Various experiments were carried out into four phases to assess the effect of different conditions into the nutrient removal and biomass production of *C. vulgaris*. It was observed that the cultivation of *C. vulgaris* under mixotrophic mode was found to be more beneficial in the bioremediation of hydroponic and aquaponic wastewater and in the production of biomass than heterotrophic and autotrophic modes of cultivation. The effect of different nitrogen to phosphorous molar ratios into nutrient removal and biomass production has also been assessed. Techno-economic assessment of microalgal biofuels has also been conducted, with a focus on the effect of different cultivation modes.

The best results in terms of total nitrogen and total phosphorous treatment efficiency were reported for mixotrophic growth supplied with 2.5 g/l glucose and atmospheric CO$_2$, showing reasonable removals of total nitrogen (TN) (98.5%), total phosphorus (TP) (99.99%) for hydroponic wastewater sample, and TN (98.5%), TP (99.9%) for aquaponic wastewater sample. The maximum biomass production and productivity were reported also for mixotrophic conditions in both hydroponic and aquaponic wastewater, showing a reasonable amount of biomass concentration (1.26 g/l) and biomass productivity (0.1108 g/l/d) for hydroponic wastewater, and biomass concentration and biomass productivity of 0.99 g/l, 0.089 g/l/d for aquaponic wastewater. Furthermore, the best results in terms of lipid content values were obtained under heterotrophic growth: 37 wt% on an Ash-free dry weight (AFDW) basis in aquaponic wastewater sample and a 33 wt% on an AFDW basis in hydroponic wastewater sample. On the other hand, the highest lipid production was obtained under mixotrophic growth (0.374 g/l) growth, followed by heterotrophic mode (0.341 g/l) in hydroponic wastewater sample.
The best treatment efficiency were reported for N:P molar ratio of 8:1, displaying removals of TP (88%) and TN (85%) compared with that for N:P ratios of 16:1 and 24:1. Maximum values with respect to biomass production was reported for N:P molar ratio of 8:1 while biomass productivity was almost the same in all N:P molar ratios. Moreover, the best results regarding the net profit were obtained for both mixotrophic and heterotrophic cultivations of 26.4 MMUS$/y (2016 US$) and 26.1 MMUS$/y (2016 US$) respectively, while the net profit for autotrophic cultivation was 4.12 MMUS$/y (2016 US$). Sensitivity analysis shows that biodiesel and nutritious supplements from soluble protein have the greatest impact on the process economics with respect to mixotrophic cultivation while biodiesel and feeds from insoluble protein have the largest effect on the process economics in connection to both heterotrophic and autotrophic cultivations.

Keywords: Wastewater Treatment, Biomass Production, Techno-economic Assessment, Egypt
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>AFDW</td>
<td>Ash-free dry weight</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphates</td>
</tr>
<tr>
<td>BOD</td>
<td>Biological Oxygen Demand</td>
</tr>
<tr>
<td>CARES</td>
<td>Center for Applied Research on the Environment and Sustainability</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>C. vulgaris</td>
<td>Chlorella vulgaris</td>
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<td>GHGs</td>
<td>Greenhouse Gases</td>
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<td>LCA</td>
<td>Life cycle analysis</td>
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<td>MBL</td>
<td>Woods Hole MBL (Marine Biological Laboratory) medium recipe</td>
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<tr>
<td>MENA</td>
<td>Middle East and North Africa</td>
</tr>
<tr>
<td>MM</td>
<td>Million</td>
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<td>N:P</td>
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<tr>
<td>OD&lt;sub&gt;680&lt;/sub&gt;</td>
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<td>PBRs</td>
<td>Photobioreactors</td>
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<tr>
<td>RIS</td>
<td>Research Institute for a Sustainable Environment</td>
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<tr>
<td>SDGs</td>
<td>Sustainable Development Goals</td>
</tr>
<tr>
<td>TCI</td>
<td>Total capital investment</td>
</tr>
<tr>
<td>TEA</td>
<td>Techno-economic assessment</td>
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<tr>
<td>TN</td>
<td>Total Nitrogen</td>
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<tr>
<td>TP</td>
<td>Total Phosphorus</td>
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Chapter 1 INTRODUCTION

1.1 Background and Motivation

In 2017, the average primary energy consumption growth was 2.2% up from 1.2% in 2016, which is considered the highest growth since 2013 (BP, 2018). Natural gas was responsible for the highest increase in the primary energy consumption while the average oil and coal consumption growth was 1.8% and 1% respectively (BP, 2018). The average growth of renewable power was the largest increase on the record: 17% (BP, 2018). Considering the ongoing technological developments in energy sector, such as the exploration of newer unconventional reserves, that fossil fuels will continue to dominate the energy market at the lowest price in comparison with other cleaner sources of energy for a certain period of time is highly possible. However, this trend will contribute directly to the man-made climate change as it is attributed primarily to greenhouse gas emission from the usage of fossil fuels and land use change. The damaging consequences for climate change
regarding the environment and human systems is tremendous, and as such hinders the global efforts towards low carbon energy systems. Due to the gradual increase in Greenhouse Gases (GHGs) emission, which is mainly because of the immense usage of fossil fuels, it has become of paramount importance to promote policies and programs in order to augment the involvement of sound and resilient environmental strategies that will minimize the effects of climate change and subsequently boost the prospects of achieving the Sustainable Development Goals (SDGs). To achieve some of these goals, a variety of technologies have been proposed, such as the reliance on cleaner sources of energy to mitigate GHGs emission.

Renewable energy technologies can play a fundamental role in addressing the issue of resource scarcity and anthropogenic climate change. One of these technologies is the development of biofuel resources that can replace the petroleum based-energy resources which will come in the light of energy security and mitigation of GHGs emission. The production of renewable source of energy from biofuels has been through different generations. The first-generation biofuels, commonly bioethanol and biodiesel, are mainly derived from oil-based crops and food, such as oilseed rap and sugarcane through the application of conventional technologies (Biofuels: prospects risks and opportunities, 2018). Although the first generation of biofuels is considered as one of the major advancements in the production of liquid biofuels, it has faced many critical opprobrium, particularly with regards to its impacts on food security (Moore, 2008). Furthermore, relying on the first generation biofuels could be a hindrance to the prerevision and protection of natural resources from depletion, which would tremendously have a harmful environmental, social, and economic ramifications (Brennan & Owende, 2010). The introduction of the second-generation biofuels, which are produced from non-food biomass, such as agricultural residues and forest harvesting residues, has been viewed as a boost since the controversy of the first generation regarding the issue of food scarcity (Moore, 2008). However, this generation also has many disadvantages, such as high land use, high water use, and not yet reaching a commercial management scales (Brennan & Owende, 2010).

Considering the cons of these generations, it is imperative to discover a sustainable source of biofuels that should be competitive and economically feasible in comparison with fossil fuels. It is also important that the newer sources should not require high land use, should mitigate CO₂
emissions, and should not require high water use. Microalgae are capable of meeting these objectives if they are exploited carefully with full consideration to cultural specifications and species selection (Suali & Sarbatly, 2012). This will play a critical role with regards to the issue of energy security while simultaneously reducing the environmental externalities (Mata et al., 2010; Sorest, 2000).

In recent years, there has been an increasing interest in the exploration of microalgae as a sustainable feedstock with regards to the production of biofuels. Microalgae are capable of producing high value bio-product and biofuels (Li, Horsman, Wu, Lan, & Dubois-calero, 2008). Among the merits of microalgae is that they are capable of producing a huge amount of oil throughout the year, and that they have a high rate in terms of the absorption and uptake of CO₂ (Brennan & Owende, 2010). In connection to land use, microalgae do not require a huge area of land to be cultivated, meaning the issue of food security will not be compromised (Xin, Hong-ying, Ke, & Ying-xue, 2010). Microalgae can be grown in aqueous media without the need for high water usage compared with terrestrial crops, thus minimizing the burden on freshwater resources (Brennan & Owende, 2010). Furthermore, the growth potential of microalgae is tremendous: the cell doubling time is in the range of 1-10 days (Schenk et al., 2008), considering the fact that many species of microalgae have high lipid content, more than 50 percent of dry weight (Hu et al., 2008). Moreover, microalgae can grow in a variety of climate conditions and water resources, in particular sea water and wastewater. Indeed, wastewater contains a considerable amount of valuable nutrients, such as nitrogen and phosphorus, that can be used to grow and cultivate microalgae. Due to the huge amount of nutrients that can be found in wastewater, microalgae could be the most plausible medium with regards to its growth in this strain. A successful utilization of nutrients from wastewater would play a fundamental role to convert both the nutrients and CO₂ into utilitarian biomass that can be later converted to different forms of renewable energy. The research presented here was conducted to determine the viability of producing microalgal biomass grown in wastewater from greenhouse farm.

Recent developments in the field of biofuels production have highlighted the need for the exploration of wastewater as the growth medium for the cultivation of microalgae in order to treat wastewater and to produce biofuels and valuable by-products. In fact, wastewater is one of the
fundamental problems besetting the environment because it contains a lot of contaminates, particularly inorganic compounds such as nitrogen and phosphorus. The fact is that removing the inorganic nitrogen and phosphorus through the use of existing wastewater treatment technologies is very difficult and costly. Therefore, the use of microalgae for the bioremediation of wastewater, fixation of CO$_2$, and production of biofuels could be the most plausible solution regarding these issues. This would play a vital role towards achieving the global efforts to reduce GHGs emission.

However, the production of microalgal biomass and bioremediation of wastewater require the study of all environmental parameters, such as light, temperature, pH, CO$_2$ and nutrients, in order to enhance the growth and lipid content of microalgae. Thus, in this study, the effects of these parameters on microalgal growth have been assessed so as to enhance the growth of the selected microalga (C. vulgaris) in greenhouse wastewater.

1.2 Possible Outcomes and Benefits

From the perspective of measures taken to contain environmental contamination, growing microalgae in hydroponic and aquaponic wastewater is expected to offer various benefits as mentioned below.

1- Bioremediation of hydroponic and aquaponic wastewater by microalgae, owing to the concurrent utilization of effluents nutrients and organic content by microalgae, will earn positive water footprint for the thesis.

2- Production of a considerable amount of biomass by cultivating microalgae in hydroponic and aquaponic wastewater will gain a positive impact in boosting the prospects of producing low carbon source of energy (bioenergy)

3- Different cultural metabolisms (Autotrophic, Heterotrophic, Mixotrophic condition) is expected to give different results regarding the growth rate of C. vulgaris, removal of nutrients, and production of biomass

4- Different Nitrogen to Phosphorous molar ratios are anticipated to have a great impact on the growth rate of C. vulgaris, removal of nutrients, and production of biomass

5- Techno-economic analysis will present variable results
1.3 Need for the Study

It is expected that around three billion people will suffer from water shortages by 2050, and a gap of 40% between sustainable water supplies and withdrawals is prognosticated to develop by 2030 (Frascari et al., 2018). Around 20% of underground water resources are overutilized throughout the world (Gleeson, Wada, Bierkens, & Van Beek, 2012), and an enrichment of water bodies by nutrient salts are anticipated to escalate by 2030 (Frascari et al., 2018). An exacerbation of water crisis due to a significant variations in conjunction with the global water resources’ availability and allocation is expected to increase as a consequence of global warming and climate change (Frascari et al., 2018). The majority of freshwater resources, 70%, are used for agricultural activities for food production (Pfister, Bayer, Koehler, & Hellweg, 2011), and a large quantity of fossil energy is used to pump irrigation water (Hodges et al. 1994). These huge water and energy consumptions are associated with a massive amount of wastewater and greenhouse gases emissions. The consequences of water shortages, poor sanitation, production of huge quantities of wastewater, and reliance on fossil fuels as a major energy source are germane in Middle East and North Africa (MENA) region. Water crisis is one of the biggest challenges that face MENA region according to the Global Risk Report published by the World Economic Forum (2019). This is expected to be exacerbated by the economic growth and population growth. Groundwater overexploitation, water quality deterioration, and unsustainable management of wastewater have significantly affected the region, a situation directly affecting socioeconomic development, predominantly in agriculture, which uses 86% of water withdrawals in MENA countries (Qadir, Bahri, Sato, & Al-Karadsheh, 2010; Varis & Abu-Zeid, 2009).

Wastewater treatment in many MENA countries is still low, around 43% (Frascari et al., 2018). Despite the small numbers of the available wastewater treatment plants (WWTPs) in some MENA countries, the treated wastewater is characterized by insufficient removal of the main contaminants. This poor removal of pollutants is due of the absence of tertiary and advanced treatments technologies, inter alia, poor maintenance and monitoring, and low qualification of personnel (Qadir et al., 2010). In addition, most of the existing WWTPs in MENA region are located mainly in major cities and rarely in small cities and suburban areas. Thus, these small cities and suburban areas are not able to effectively treat the wastewater. Consequently, the wastewater
load will be disposed to the environment, resulting in environmental pollution. Decentralization of wastewater treatment might be one of the plausible solutions to such problem. Anaerobic filter, septic tank, anaerobic and aerobic pond are some of the existing decentralized technologies (Massoud et al. 2009). Nevertheless, activated sludge process is widely used in major cities, and it is very difficult to be employed within a community scale due to its complication and its concomitant costs. Thus, there is a paramount need for developing a sustainable on-site wastewater treatment technology to be employed at the community level.

To scale up commercially, there is a need for a better understanding of the potential benefits of the utilization of microalgae for bioremediation of wastewater and production of biomass, and for a structured approach in exploring and modeling the economic implications regarding the use of microalgae cultivation with wastewater for sustainable production of biofuels. In this respect, very few studies, according to the author, have been undertaken to evaluate the use of microalgae to bioremediate wastewater from greenhouse farms (hydroponic and aquaponic wastewater) and to produce biomass although numerous studies have studied the use of microalgae for treating different types of wastewater. Specifically, the following research questions need to be addressed:

1- Is wastewater, in this research study hydroponic and aquaponic wastewater, a practical replacement for microalgae's nutrient and/or water requirements?
2- Are microalgae a plausible solution for bioremediating wastewater in terms of nutrients from hydroponic and aquaponic wastewater?
3- Are microalgae capable of producing a considerable amount of biomass under wastewater conditions that would be suitable for producing bioenergy?
4- What are the characteristics of studies that can evaluate the economic costs and benefits of systems that span the use of wastewater for biomass production through the utilization of microalgae?
1.4 Objectives

One of the primary focus of this research was to determine the removal efficiency of nutrients and to evaluate the biomass production in wastewater from greenhouse farm using *C. vulgaris*. How different cultural conditions that affect the treatment efficiency of wastewater and biomass production has also been assessed. This study was conducted in order to contribute to the recent development in the production of biofuels from microalgae, mainly regarding the issue of wastewater treatment and production of biomass. Thus, the ultimate objective of this study was to enhance the growth of the selected microalgae strain (*C. vulgaris*) in order to remove the nutrients from greenhouse farm wastewater and to produce biomass. The specific objectives of this research were:

1- Adjusting the conditions in wastewater from hydroponic and aquaponic wastewater to grow *C. vulgaris*
2- Determining the removal efficiency of Nitrogen and Phosphorus in wastewater from hydroponic and aquaponic farms using *C. vulgaris*
3- Examining the Effect of different cultural metabolisms (Autotrophic, Heterotrophic, Mixotrophic condition) into the Growth of *C. vulgaris*, Nutrients Uptake, and Biomass Production
4- Assessing the Effect of Different Nitrogen to Phosphorus (N:P) Molar Ratios into the Growth of *C. vulgaris*, Nutrients Uptake, and Biomass Production
5- Examining the effect of non-sterilized hydroponic and aquaponic wastewater under mixotrophic conditions
6- Conducting a Techno-economic analysis with regards to the use of *C. vulgaris* in wastewater and production of biofuels
1.5 Structure of the Thesis

This master’s thesis is composed of five themed chapters. Chapter one covers the introduction on background and motivation, problem definition, scope and objectives, possible benefits and outcomes of the study. Chapter 2 begins by laying out the theoretical dimensions of the research and looks at different case studies relevant to the research topic. Materials and methods with regard to different experimental processes that have been conducted in the research work are explained in chapter 3. The fourth chapter presents the findings and discussion for the experimental work. Techno-economic assessment is presented in chapter five, focusing on three themes: background and motivation, methodology, and results and discussion. Chapter 6 gives conclusion with summary and recommendations for future work.
Chapter 2 LITERATURE REVIEW

2.1 Background

This chapter provides a review of the literature regarding microalgae cultivation in wastewater, including factors affecting the growth rate of microalgae, nutrients (nitrogen and phosphorus) removal mechanisms and microalgal biomass production technologies.
2.2 Microalgae

Microalgae are single-celled organisms that exist in the marine as well as freshwater sources. They have photosynthetic characteristics analogous to territorial plants. Since microalgae cellular structures are simple and exist in an aqueous environment that gives them the efficient utilization of water, CO\textsubscript{2} and nutrients, their efficiency regarding the conversion of solar energy into biomass is tremendous (Gouveia, 2011). Some examples of microalgae are prokaryotic microalgae (cyanobacteria), eukaryotic microalgae (green algae, red algae, and diatoms) (Mata et al., 2010). Moreover, microalgae are one of the fundamental natural resources in the earth due to the fact that they play a big role with regards to photosynthetic carbon assimilation. It is estimated that microalgae contribution as for the global photosynthetic activity is 50 percent (Chiu et al., 2015). According to Richmond (2004), a tremendous number of microalgae species exist – more than 50,000 species; however, a limited number of them have been studied and examined – about 30,000 species (Richmond, 2004). Among these, Chlorella species are the most exploited microalgae for the production of biofuels and bioremediation of wastewater.

The potential of microalgae to produce valuable products that can be utilized for different purposes is scientifically acknowledged. Proteins, lipids, carbohydrates, carotenoids or vitamins for health, food and feed additives, cosmetics, and the production of renewable energy are some examples of the products that can be produced by microalgae. Historically, Chinese were the first to have used microalgae (Nostoc) 200 years ago in order to survive during famine (Priyadarshani & Rath, 2012). Nevertheless, the serious exploration and development regarding the biotechnology of microalgae started in the middle of the last century. In early 1960s, the first large-scale culture regarding microalgae has been accomplished in Japan with the culture of Chlorella by Nihon Chlorella while the use of microalgae to produce a renewable source of energy has gained great attention during the first oil crisis in 1970s (Spolaore et al., 2006).

Currently a tremendous number of applications regarding microalgae have been explored. Microalgae serve as good nutritional source for human because they are rich source of carbohydrates, protein, enzymes, fiber, and many minerals (vitamins). Another important benefit of microalgae is that many of their components are commonly used in cosmetics for different
purposes, for example as thickening agents, water-binding agents, and antioxidants. Arthrospira and Chlorella are the common types of microalgae that are used in skin care market. Microalgae also can be used to both treat wastewater and produce biofuels (Priyadarshani & Rath, 2012). They are useful for a variety of animals such as fish (aquaculture), pets, and farm animals as they can be incorporated into the feed of these animals (Spolaore et al., 2006).

Microalgae is considered to be the third generation with regards to the production of biofuels and mitigation of CO₂. The fact is that microalgae are capable of mitigating CO₂ biologically through the conversion of CO₂ to organic matter. Microalgal biomass can be converted to produce renewable sources of energy, such as biodiesel, biogas, and biohydrogen through different conversion methods, which can be classified into biochemical conversion, chemical reaction, direct combustion, and thermochemical conversion. Microalgae are also a fundamental source for the treatment of wastewater since the former contains numerous nutrients and heavy metals which microalgae can use for their uptake (Shaikh Abdur Razzak, Ali, Hossain, & deLasa, 2017).

2.3 The Logic Behind the Selection of C. vulgaris

The primary stage in the bioremediation of wastewater and production of biofuels using microalgae is the selection of microalgal strain. An expeditious selection of microalgal species with properties conforming to cultural conditions and products will ensure a full utilization of comparative advantage. Microalgae are diverse species, making the selection process difficult. Most microalgae are capable of growing in a variety of cultural metabolisms (autotrophy, heterotrophy, mixotrophy and photoheterotrophy) according to carbon source and energy source.

Much of the current literature on microalgae pays particular attention to the species having the capability to quickly grow in wastewater, having resistance and tolerance against contamination, and having the ability to accumulate a considerable amount of lipid and biomass. Chlorella vulgaris, Scenedesmus dimorphous, Dunaliella salina , and Botryococcus braunii are some examples of the species that are currently used for bioremediation of wastewater and production of bioenergy (Lam et al., 2017; Luangpipat & Chisti, 2017; Salgueiro, Pérez, Maceiras, Sánchez, & Cancela, 2016; Znad, Al Ketife, Judd, AlMomani, & Vuthaluru, 2018).
C. vulgaris is the most exploited microalgae for the production of bioenergy and treatment of wastewater since it has the ability to remove not only the nutrient but also heavy metals (Lam et al., 2017; Lau, Tam, & Wong, 1997; Otando, Kokabian, Stuart-Dahl, & Gude, 2018). C. vulgaris also is very strong, and has good tolerance to a wide variety of chemical parameters (carbon, nitrogen and phosphorus) and physical factors (light, pH, and temperature) (Dehaghani & Pirouzfar, 2018). Most importantly, C. vulgaris is capable of growing in all of the aforementioned growth metabolisms. Furthermore, the strain also accumulates a considerable amount of lipid [32.7 wt% on an ash-free dry weight basis “AFDW”] (Lam et al., 2017). These properties make the strain suitable for the treatment of wastewater and production of biofuels.

2.4 Microalgal Biomass Production Technologies

One of the fundamental issues regarding the enhancement of both microalgal biomass and removal efficiency of nutrients is the selection of the appropriate microalgal cultivation systems. In fact, a considerable amount of research has been done as for microalgae cultivation systems. Microalgal cultivation systems are categorized according to the cost, the type of the desired products, nutritional source, and mitigation of CO₂ as open systems or closed system as well as Hybrid systems (Shaikh A. Razzak, Hossain, Lucky, Bassi, & De Lasa, 2013).

2.4.1 Open pond systems

Open pond cultivation systems have been widely used regarding the culturing of microalgae. They are outdoor facilities which can be classified into natural water, such as lakes, lagoons and ponds, and artificial systems, such as artificial ponds, containers, and tanks (Brennan & Owende, 2010). A variety of configurations in connection to open pond systems have been developed for the sake of enhancing the biomass production and the treatment efficiency of wastewater. Commonly, the raceway pond is the most artificial system that has been used for the cultivation of microalgae. This type of ponds are made of a closed loop or oval recirculation channels (Fig. 2.1) (Shaikh Abdur Razzak et al., 2017). The depth of this type is usually ranged between 0.2 to 0.5 meter, and they are constructed by using concrete materials (Brennan & Owende, 2010). It is designed in a way that a continuous supply of CO₂ and nutrients are provided through recirculation of algal
culture. Also, in order to hinder the sedimentation, a reasonable mixing is provided with a paddle wheel (Shaikh Abdur Razzak et al., 2017). Table 2.1 shows the merits and de-merits of open pond systems.

**Table 2.1: Merits and de-merits of open pond systems**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheaper methods regarding large scale biomass production (Schenk et al., 2008)</td>
<td>Poor light utilization due to the fluctuations of weather conditions (Makhdoomi et al., 2010)</td>
</tr>
<tr>
<td>Easy to clean (Schenk et al., 2008)</td>
<td>Lower biomass productivity (Shaikh Abdur Razzak et al., 2017)</td>
</tr>
<tr>
<td>Lower operational cost and good use of sunlight (McGinn et al., 2011)</td>
<td>A huge area of land is required (Shaikh Abdur Razzak et al., 2017)</td>
</tr>
<tr>
<td>Lower energy inputs (Brennan &amp; Owende, 2010)</td>
<td>Contamination problems due to the competition of other microorganisms with microalgae for food (Shaikh Abdur Razzak et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>A limited number of microalgal species can be grown (Harun, Singh, Forde, &amp; Danquah, 2010)</td>
</tr>
<tr>
<td></td>
<td>Limited amount of CO₂ required for photosynthesis due to the reliance on CO₂ in the atmosphere (Shaikh Abdur Razzak et al., 2017)</td>
</tr>
</tbody>
</table>
2.4.2 Closed systems

Due to many problematic issues that are associated with the open systems cultivation of microalgae, closed systems or photobioreactors (PBRs) technologies have been developed in order to overcome these problems. Various closed systems have been developed in the recent years mainly for the sake of light optimization and other important issues, such as costing, selection of the materials, production scale, and operational conditions (CO₂ bubbling, removal of oxygen, temperature, pH, nutrients etc.) Tubular, cylindrical, and flat panel systems are some examples of the available PBR technologies (Bux & Chisti, 2016). Figure 2.2 shows a nonstop recirculation of water and algae with regards to PBRs. The major advantages of PBRs are that they are capable of preventing other types of contaminant microorganisms that can compete with microalgae; unlike open system photobioreactors can be operated under various weather conditions; due to the mixing control, the issue of CO₂ losses is tremendously reduced; regarding temperature adjustment, it can be regulated easily through the placement of the reactor into a room in which the temperature can be controlled (Shaikh Abdur Razzak et al., 2017). Another important advantage also is that CO₂ can be captured from the flue gases with a considerable amount of CO₂ concentration (Shaikh Abdur Razzak et al., 2017; B. Wang, Li, Wu, & Lan, 2008).
Although PBRs have several merits that outweigh open system technologies, they have some limitations that are related to the principles of their operation. There is a great risk regarding the formation of biofilm due to the culture confinement, and this can cause the accumulation of oxygen in the culture which directly affects the photosynthetic efficiency. Overheating of the culture also is a possible problem that can occur because of the tremendous amount of infrared radiation that might be absorbed by the culture medium. Various solutions have been proposed in order to overcome those limitations. For example, biofilm formation can be overcome by optimizing mixing conditions, particularly through increasing both heat and gas–liquid mass transfer. Also, temperature can be controlled significantly indoor instead of putting reactor outdoor. The only challenge of PBRs is the light control which is very important to be optimized because its fundamental with regards to the productivity of the system (Bux & Chisti, 2016; Carvalho, Silva, Baptista, & Malcata, 2011; Grobbelaar, 2009).

![Diagram of PBRs](image)

**Figure 2.2:** A nonstop recirculation of water and algae with regards to PBRs (Shaikh Abdur Razzak et al., 2017)
2.5 Microalgae Culture Conditions

One of the good features of microalgae is that they have a tremendous ability to grow in a variety of cultural metabolisms in accordance with the carbon source and the energy source. According to this feature, microalgae can grow on the strength of four types of cell metabolisms: autotrophy, heterotrophy, mixotrophy and photoheterotrophy (Richmond, 2013).

2.5.1 Autotrophic conditions

In autotrophic cultivation, microalgae absorb light energy so as to reduce CO$_2$ by the oxidation of substrates (commonly water) with the discharge of oxygen. In other words, microalgae use light energy as their energy source while they use inorganic carbon as their carbon source (Richmond, 2013). Autotrophic cultivation is the widely used cultivation condition regarding the growth of microalgae (Brennan & Owende, 2010; C. Y. Chen, Yeh, Aisyah, Lee, & Chang, 2011). Data from several studies suggest, based on the type of microalgae, that the lipid content of microalgae ranges from 5% to 68% under phototrophic conditions (C. Y. Chen et al., 2011). There is a consensus in the literature that increasing the lipid content of microalgae is usually achieved at the expense of nitrogen or nutrient limitation (C. Y. Chen et al., 2011). This indicates that the achievement of higher lipid content leads to lower biomass productivity, demonstrating the fact that the oil-producing ability of microalgae is not measured based on the lipid content. Therefore, it is of paramount importance to consider concurrently the effect of both lipid content and biomass production when it comes to the oil productivity of microalgae. Accordingly, lipid productivity (effect of lipid content plus biomass production) represents an appropriate performance index with regards to the oil-producing ability of microalgae (Brennan & Owende, 2010; C. Y. Chen et al., 2011). According to Chiu et al. (2008), under phototrophic cultivation using 2% CO$_2$ with 0.25 vvm aeration, Chlorella sp. have the highest lipid productivity, which is about 179 mg/L/d (Chiu et al., 2008).

One of the major advantages of autotrophic cultivation is the utilization of light as energy source and inorganic carbon as carbon source which are very important regarding the growth of microalgal cells and oil production simultaneously. Nevertheless, insufficient light intensity plus CO$_2$ supply are the twin problems for phototrophic culture (Suali & Sarbatly, 2012). In outdoor
growth, photosynthesis efficiency is limited to the time when light is present (Suali & Sarbatly, 2012) while the site of microalgae cultivation should be approximate to power plants for the sake of carbon source supply (C. Y. Chen et al., 2011). Furthermore, unequal distribution of light among the microalgal cells significantly affects the productivity (Suali & Sarbatly, 2012). Open systems, such as raceway ponds are the most operated cultivation systems under phototrophic cultivation conditions (C. Y. Chen et al., 2011).

2.5.2 Heterotrophic Cultivation

In heterotrophic cultivation, microalgae reproduce by using organic carbon, instead of inorganic carbon, substance as their carbon source and energy source; therefore, this cultivation does not depend on the light source to reproduce (Shaikh Abdur Razzak et al., 2017). In comparison with other cultivation conditions, heterotrophic culture has the highest lipid productivity. For example, lipid productivity under heterotrophic cultivation is about 20 times that of photoautotrophic cultivation (Shaikh Abdur Razzak et al., 2017). A 40% increase in lipid content is reported in Chlorella protothecoides through the replacement of cultivation condition from phototrophic to heterotrophic (C. Y. Chen et al., 2011). Because heterotrophic microalgae’s cells growth do not depend on light, heterotrophic cultivation is considered less expensive when it comes to the growth of the cells. Also, it is expected that heterotrophic cultivation might be the most plausible option regarding the bioremediation of large volumes of wastewater effluents (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011a). However, heterotrophic cultures have several disadvantages: inability of all species of microalgae to grow under heterotrophic conditions; increasing energy expenses and cost due to the addition of an organic matter, and contamination problems from the competition with other microorganisms (Perez-Garcia et al., 2011a). According to these limitations, microalgal cultivation under heterotrophic conditions is not considered to be practical for the production of biofuel, particularly due to purchasing of carbon source, as it increases the production cost (Suali & Sarbatly, 2012).

2.5.3 Mixotrophic Cultivation

In mixotrophic culture, microalgae undergo photosynthesis, and both organic carbon and inorganic carbon (CO$_2$) are used as a carbon source as for the microalgal growth. This indicates that
microalgae are capable of accustoming to both cultures, phototrophic or heterotrophic conditions. In mixotrophic cultivation, the carbon source is obtained from both organic matter and CO₂; then the CO₂ released via respiration by microalgae will be trapped and reused under phototrophic culture. Mixotrophic cultivation is infrequently used in microalgal oil production in comparison to phototrophic and heterotrophic cultivation (C. Y. Chen et al., 2011).

2.5.4 Photoheterotrophic Cultivation

In photoheterotrophic culture, microalgae use light as their energy source while they use organic compounds as their carbon source. Photoheterotrophic and mixotrophic culture are generally alike as they can be described based on the energy difference that is required to make the growth and specific metabolite production (Mata et al., 2010). Although some of light-regulated beneficial metabolites can be produced using photoheterotrophic cultivation, using this cultivation in the production of biodiesel is infrequent (C. Y. Chen et al., 2011).

2.6 Factors Affecting the Growth Rate of Microalgae

It is critical to control the growth conditions of microalgae in order to enhance the removal efficiency of nutrients in wastewater and to facilitate the process of biomass production. Chemical parameters, such as carbon, nitrogen and phosphorus, are the main parameters that affect the microalgal growth. Additionally, physical factors, such as light, pH, and temperature are of paramount importance to be controlled.

2.6.1 Carbon

Carbon constitutes approximately around 50% of the microalgal cell mass (Bux & Chisti, 2016). According to stoichiometric formula of most of the algal cell, about 1.83 tons of inorganic CO₂ are required to produce one tone of algal biomass (Chisti, 2007). During the process of photosynthesis, inorganic compounds are assimilated by microalgae and are converted to chemical energy, which will later be converted to starch and oils (Larsdotter, 2006; Shaikh Abdur Razzak et al., 2017). Inorganic CO₂ can be provided through aeration to microalgal medium; however, due to the low ambient atmospheric concentration (0.033%), an additional CO₂ must be provided in
order to sustain the microalgal growth (Larsdotter, 2006). This is commonly done by feeding air with 1-5% CO₂ to the culture medium (Larsdotter, 2006). Furthermore, organic carbon sources, such as glucose and glycerol, can be used as a carbon source during heterotrophic or mixotrophic growth (Larsdotter, 2006; Perez-Garcia et al., 2011a).

2.6.2 Nitrogen and Phosphorus

Nitrogen is one of the major nutrient constituents regarding microalgae, and it encompasses of more than 10% of microalgal biomass (Larsdotter, 2006). Ammonium and nitrate are the highest driving factors of nitrogen compounds that play a fundamental role in microalgal growth. Ammonium, in particular, is the most preferable one for the growth of microalgal cells. Urea and nitrite can also be utilized as nitrogen sources; however, they are toxic, particularly when they are utilized in high concentrations. In a study conducted by Silva et al. (2015) to specify the best source of nitrogen for microalgae C. vulgaris and Pseudokirchneriella subcapitata, ammonium was the preferable source of nitrogen for microalgae C. vulgaris (Silva et al., 2015). Although, microalgal cells prefer ammonium for their growth, it can be harmful and toxic if it is presented in higher concentration (Larsdotter, 2006; Shaikh Abdur Razzak et al., 2017). To avoid such harmful effect, ammonium concentration less than 20 mg/liter is recommended (Larsdotter, 2006). Limitation of nitrogen can cause discoloration of microalgal cells (reduction of chlorophylls and carotenoids increase) and accumulation of organic compounds, such as some oils (Goiris et al., 2015; Richmond, 2004).

Phosphorus also is another important macro-nutrient that is taken by microalgae to enhance their growth as orthophosphate (PO4-3) (Larsdotter, 2006). Microalgae have the ability of converting inorganic phosphorus constituents to organic constituents by incorporating them through phosphorylation. Furthermore, some microalgal species are capable of using phosphorus that can be found in organic esters, which is vital regarding microalgal growth (Cai, Park, & Li, 2013a). Microalgal cells store the excesed amount of phosphorus as polyphosphate granules, which can be utilized later during the starvation of phosphate. Reduction of phosphates might have a negative effect for the process of photosynthesis as well as for lipid productivity (Shaikh Abdur Razzak et al., 2017).
The ratio between nitrogen and phosphorus (N:P) also is fundamental because it affects both the biomass productivity and the maintenance of the dominance species in the culture (Richmond, 2004). Silva et al. (2015) estimated the effect of different N:P ratios (8:1, 16:1, and 24:1) on the growth of microalgae C. vulgaris and P. subcapitata. The most preferred ratio for the growth of microalgae C. vulgaris was 8:1 (Silva et al., 2015). Furthermore, it has been suggested that the N:P ratio of 30:1 indicates phosphorus limitation while 5:1 indicates nitrogen limitation (Larsdotter, 2006).

2.6.3 Light Intensity and Temperature

An effective utilization of light is one of the important parameters that enhances the growth rate of microalgal cells. Autotrophic microalgae obtain light from energy while some microalgae can grow heterotrophically by utilizing organic matter as both energy and carbon source. During the process of photosynthesis, microalgae need light to grow efficiently. Photooxidation might happen if the light is not provided sufficiently to microalgal culture. In addition, photoinhibition may occur if the light is provided tremendously. Many recent studies (Bux & Chisti, 2016; Larsdotter, 2006) have demonstrated the important factor light plays in inhibiting microalgal growth. There are several reasons to such inhabitation, among them is shading phenomena, a phenomenon in which some microalgal cells do not receive enough light due to the high density of microalgal cells that are present in the upper zone. Creating turbulence is one of the good methods that are used to avoid such problem. Another solution is the decreasing of cultural depth of the vessel. Accordingly, depth between 15-20 is appropriate for microalgae to get enough light (Larsdotter, 2006).

Another crucial issue is that the amount of light energy received by microalgae, which is directly correlated with photon flux density that hit the surface of the culture (Richmond, 2004). A frication of the photon flux is absorbed by the cells according to many factors like the density of the cells, the optical properties of the cells, and the degree of the mixing. The photons that are not absorbed by the cells will be dissipated as heat (Jacob-Lopes Leila Queiroz Zepka Maria Isabel Queiroz Editors, n.d.).
It has been demonstrated that there is a great interaction between light and temperature, suggesting that increasing light intensity will increase the optimum temperature for photosynthesis (Jacob-Lopes Leila Queiroz Zepka Maria Isabel Queiroz Editors, n.d.; Larsdotter, 2006). In a study conducted by Gonçalves et al. (2016) to examine the effect of light and temperature on the growth of some microalgae species, including *C. vulgaris*, and nutrients removal, one of the findings was that both optimum temperature, 25 °C, and optimum daily irradiance, 208 µmol/m²/s, are obtained in the case of *C. vulgaris*. Furthermore, temperature increase plays a big role in the growth of microalgae; however, over-increasing temperature can cause a rapid decrease in microalgal growth owing to photoinhibition. Conversely, at lower temperature, microalgae can be photo-inhibited by high light intensity. In general, temperature between 15-25 °C is suitable for most of the microalgae (Larsdotter, 2006).

2.6.4 pH

During the photosynthetic assimilation of CO₂, a steady increase in pH occurs due to the accumulation of the hydroxide ion (OH⁻) in the culture medium (Richmond, 2004). This accelerates the formation of carbonates (CO₃²⁻) in the culture medium, which is not preferable for most microalgae as a source of carbon. On the contrary, a decrease in the pH of the solution changes the chemical equilibrium, thereby accelerating the formation of CO₂, which is the preferable sources of carbon as for microalgae. However, providing tremendous amount of this could inhibit the growth of the microalgae (Larsdotter, 2006).

Nitrogen also affects pH in the medium. PH of a culture that contains nitrate ions have a tendency of increasing pH as a result of proton H⁺ removal while a culture medium that contains ammonia tends to decrease pH due to the accumulation of H⁺ (Bux & Chisti, 2016; Larsdotter, 2006). Furthermore, high pH value influences phosphorus concentration in microalgae, which can cause the precipitation of phosphate via the formation of calcium phosphates, and therefore limiting the availability of phosphorus for microalgae (Jacob-Lopes Leila Queiroz Zepka Maria Isabel Queiroz Editors, n.d.; Larsdotter, 2006). Higher concentration of ammonia in line with high pH can undermine the photosynthesis efficiency. Also, high pH can cause floculation of microalgae, leading to reduction of microalgal growth and nutrient uptake (Larsdotter, 2006).
2.7 Bioremediation of Wastewater Using Microalgae

Microalgae represent a sustainable source as regards to the process of phyco-remediation, a process which entails the utilization of microalgae for the sake of the contaminant’s removal from wastewater (Bux & Chisti, 2016). This process has been introduced by John to refer to the treatment of wastewater carried out by algae. The use of wastewater with respect to microalgae culture is a utilitarian approach for the minimization of freshwater resources use, reduction of the cost of chemical and nutrient addition, removal of nutrients (namely nitrogen and phosphorus), and production of biomass that can be used to produce various forms of bioenergy and useful by-products. Microalgae have high ability for the absorption of nutrients (C, N, P) as they use them for their uptakes (proteins), reduction of chemical and biological oxygen demand, and other contaminants (Abdel-Raouf, Al-Homaidan, & Ibraheem, 2012; Rawat, Ranjith Kumar, Mutanda, & Bux, 2011). In addition, microalgae can be used to bioremediate different types of wastewater including, among others, municipal wastewater, agricultural wastewater, agro-industrial wastewater, and human sewage (Cai, Park, & Li, 2013b). Some of the major merits that microalgae offer over conventional wastewater treatment techniques are: lower energy requirements, lower sludge formation, CO₂ mitigation, reducing the burden for the freshwater resources and land use, and a simultaneous production of energy-rich microalgal biomass that can be further processed for numerous applications, such as biofuels production and useful by-products (Batista et al., 2015; Bux & Chisti, 2016; Suali & Sarbatly, 2012).

2.7.1 Carbon Removal Mechanism

Microalgae have the ability of converting inorganic carbon (CO₂) into biomass through the use of the electrons released during the light-dependent water photolysis as exemplified in (Eq. 1). In this regards, carbon constitutes approximately around 50% of the microalgal cell mass (Bux & Chisti, 2016). According to stoichiometric formula of most of the algal cell, about 1.83 tons of inorganic CO₂ are required to produce one tone of algal biomass (Chisti, 2007). Many microalgae species have good tolerance to CO₂ concentration of up to 50% (v/v) which have been reported for *C. vulgaris* and *Scenedesmus obliquus* (Arbib, Ruiz, Álvarez-Díaz, Garrido-Pérez, & Perales, 2014). As a result of this high tolerance, the conversion efficiency of CO₂ is between 10 to 50 times higher in comparison with terrestrial plants (Li et al., 2008). Furthermore, organic carbon sources, such
as glucose and glycerol, can be used as a carbon and energy source with respect to microalgae growth in the absence of photosynthesis which are very useful during heterotrophic or mixotrophic growth of microalgae (Larsdotter, 2006; Perez-Garcia et al., 2011a). During wastewater treatment operation owing to the assimilation of not only wastewater alkalinity but also the CO₂ released from the oxidation of organic matter in combination with microalgal and bacterial heterotrophic metabolism, the assimilation of nutrients from wastewater is highly possible (Posadas et al., 2017). In addition, the in-situ generation of dissolved oxygen in microalgae cultivation system can accelerate both the organic matter and ammonium oxidation present in the wastewater and subsequently reducing the operational cost of wastewater treatment plant associated with the mechanical O₂ supply in activated sludge process – up to 50% of total operation cost – and minimizing the stripping of the harmful contaminants conjoined with mechanical aeration (Alcántara et al., 2015).

\[
\text{CO}_2 + H_2O + \text{nutrients} \rightarrow O_2 + \text{biomass} + \text{waste heat}
\]

2.7.2 Nitrogen Removal Mechanism

Nitrogen is one of the most widely nutrients present excessively in wastewater as a consequence of a variety of man-made activities. Nitrogen can be present mostly in the form of ammonia, but also it can be found in other forms, such as nitrate, nitrite, or organic nitrogen. These different forms of nitrogen are toxic, particularly when they are present in high concentrations, and they may cause eutrophication (Silva et al., 2015). Therefore, a sustainable removal of nitrogen from wastewater is paramount to reduce its negative impacts on aquatic life and humans. In fact, nitrogen encompasses of more than 10% of microalgal biomass which entails an enormous potential of its removal from wastewater (Larsdotter, 2006). The issue is that microalgae are capable of assimilating different forms of inorganic nitrogen present in wastewater by converting them to various organic nitrogen species needed for microalgal cell growth (Cai et al., 2013b). Nitrate and nitrite are reduced to ammonium inside microalgal cells if they present in wastewater. In fact, this reduction is facilitated by different enzymes and involved several in-between products throughout reduction pathways (Bux & Chisti, 2016). These pathways start with the reduction of nitrate to nitrite facilitated by nitrate reductase enzyme followed by the reduction of nitrite to
ammonium by nitrite reductase (Bux & Chisti, 2016; Cai et al., 2013b). Moreover, as a result of pH increase with respect to microalgae cultivation, an indirect removal of nitrogen in the form of ammonia stripping happens (García, Mujeriego, & Hernández-Maríné, 2000).

2.7.3 Phosphorus Removal Mechanism

Phosphorus also is another prevailing nutrient in raw wastewater owing to the anthropogenic activities, specifically with regards to the tremendous usage of phosphorus fertilizers in agriculture. Phosphorus can be found predominantly in form of phosphates, such as orthophosphate, polyphosphate, or organic phosphate, and its availability varies according to the chemical speciation (Bux & Chisti, 2016). Eutrophication occurs if phosphorus is present in water bodies; therefore removal of phosphorus from wastewater is of great importance to obviate such problem (Abdel-Raouf et al., 2012). Furthermore, microalgae have the ability of converting inorganic phosphorus constituents, mainly orthophosphate as \( \text{HPO}_4^{2-} \) and \( \text{H}_2\text{PO}_4^- \), to organic constituents by incorporating them through phosphorylation. Such ability is very essential for energy transfer because it plays a fundamental role with respect to the formation of adenosine triphosphates (ATP) and adenosine diphosphate (ADP) during various metabolic activities (Conley et al., 2009). Furthermore, some microalgae species are capable of using phosphorus that can be found in organic esters, which is vital regarding microalgal growth (Cai et al., 2013a). Microalgal cells store the excessed amount of phosphorus as polyphosphate granules, which can be utilized later during the starvation of phosphate (Shaikh Abdur Razzak et al., 2017). High pH value influences phosphorus concentration in microalgae, which can cause the precipitation of phosphate via the formation of calcium phosphates. This indicates an indirect phosphorus removal (Jacob-Lopes Leila Queiroz Zepka Maria Isabel Queiroz Editors, n.d.; Larsdotter, 2006).
Chapter 3 MATERIALS AND METHODS

3.1 General

The current chapter gives a full exposition of the materials and methods that have been used and followed for various experimental runs in the current research work. Thus, the microalgae, cultural cultivation protocols, experimental runs, analytical measurements and calculation equations are described.
3.2 Microalgae

The microalgae used in the present study is freshwater green algae *Chlorella vulgaris* (*C. vulgaris*) for the treatment of greenhouse (hydroponic and aquaponic) wastewater samples.

3.3 Preparation of culture medium and *C. vulgaris* inoculum

*C. vulgaris* was supplied by the Department of Biology at the American University in Cairo (Fig. 3.1). The strain was routinely cultured in MBL for long time storage. Woods Hole MBL medium recipe as recommended for freshwater microalgae was used in order to preserve the microalgae strain *C. vulgaris* in 500 ml flasks at 25°C (Fig. 3.2). *C. vulgaris* was initially added into a 10 percent (*V_inoculated/V_of_the_medium*) in 500 ml flask containing 250 ml of synthetic medium (MBL). The initial pH of the culture was adjusted between 7 to 8 by using NaOH (base) or HCl (acid) while simultaneously using pH meter. Prior to microalgae inculcation, the culture media was autoclaved for 15 min and 120°C for the sake of sterilization. The experiment (inculcation stage) was performed under stationary conditions in which temperature was 22 (+/-3) °C and light intensity of 80 mol m\(^{-2}\) s\(^{-1}\). The inoculation stage was performed under a light regime of 16:8 (light: dark). The synthetic growth medium MBL contained macro-nutrients (per liter DI) as it is shown in Table 3.1.

![Figure 3.1: C. vulgaris culture](image)
Figure 3.2: C. vulgaris grown in two cylindrical glass bottles containing MBL medium recipe

Table 3.1: Woods Hole MBL medium recipe (Nichols, 1973)

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Per litre distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CaCl₂·2H₂O</td>
<td>36.76 g</td>
</tr>
<tr>
<td>2. MgSO₄·7H₂O</td>
<td>36.97 g</td>
</tr>
<tr>
<td>3. NaHCO₃</td>
<td>12.60 g</td>
</tr>
<tr>
<td>4. KH₂PO₄</td>
<td>8.71 g</td>
</tr>
<tr>
<td>5. NaNO₃</td>
<td>85.01 g</td>
</tr>
<tr>
<td>6. Na₂SiO₃·9H₂O</td>
<td>28.42 g</td>
</tr>
<tr>
<td>7. Na₂EDTA</td>
<td>4.36 g</td>
</tr>
<tr>
<td>8. FeCl₂·6H₂O</td>
<td>3.15 g</td>
</tr>
<tr>
<td>9. Metal Mix</td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.022 g</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.18 g</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.006 g</td>
</tr>
<tr>
<td>10. Vitamin stock</td>
<td></td>
</tr>
<tr>
<td>Cyanocobalamin (Vitamin B12)</td>
<td>0.0005 g / L dH₂O</td>
</tr>
<tr>
<td>Thiamine HCl (Vitamin B1)</td>
<td>0.10 g / L dH₂O</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.0005 g / L dH₂O</td>
</tr>
<tr>
<td>11. Tris stock</td>
<td>250.0 g / L dH₂O</td>
</tr>
</tbody>
</table>

Add each constituent separately to ~750mL of distilled H₂O, fully dissolving between additions. Finally make up to 1L with distilled H₂O.
3.4 Wastewater sampling

For this study, two different sources of greenhouse farm (hydroponic and aquaponic) wastewater were used in order to monitor the growth of *C. vulgaris* and to determine both the removal efficiency of nutrient and the production of biomass. These sources were collected from the Center for Applied Research on the Environment and Sustainability (CARES) at The American University in Cairo (AUC) (Fig. 3.3). CARES is established for the sake of promotion of the sustainability research in Egypt, the Middle East, and North Africa, and for the purpose of strengthening the role that AUC plays to accelerate and promote the ideas of sustainable development research and education. Hydroponic and aquaponic wastewater samples were stored immediately at 4°C and filtered to separate out the suspended particles. Furthermore, wastewater samples were sterilized before inoculating *C. vulgaris* to kill any microorganisms that could be present in the wastewater (phase 1 to 3). In addition, the wastewater samples were analyzed to determine Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), nutrients composition (namely nitrogen and phosphorus), before using *C. vulgaris* to bioremediate hydroponic and aquaponic wastewater at the Agriculture Research Center at the University of Cairo.

![Aquaponic and Hydroponic raw wastewater samples: (A) aquaponic wastewater (B) hydroponic wastewater](image)

**Figure 3.3:** Aquaponic and Hydroponic raw wastewater samples: (A) aquaponic wastewater (B) hydroponic wastewater
3.5 Experimental Design

The experiments was designed into four phases in order to study a variety of parameters that affect the growth of *C. vulgaris*, the removal of nutrients, and the production of biomass. Each phase includes a number of reactors as it is described in the following next sections.

3.5.1 Phase 1 (Initial Phase): Evaluation of *C. vulgaris* growth, Nutrients Uptake, and Biomass Production using Hydroponic and Aquaponic Wastewater Samples and MBL

This stage served as an initial stage. The aim of this phase was to examine to what degree *C. vulgaris* grows and removes nitrogen and phosphorous in hydroponic and aquaponic wastewater samples and to compare its growth in these samples with the standard growth medium for green algae (MBL). Three tests were conducted concurrently for 23 days. For each test, *C. vulgaris* was initially added in a 10 percent \( \frac{V_{\text{inoculated}}}{V_{\text{of the medium}}} \) in three Erlenmeyer flasks (500 ml) containing 400 ml of the tested medium (MBL, hydroponic wastewater, aquaponic wastewater). Tests were employed under stationary condition where pH of the medium was adjusted between 7 to 9, and the temperature was in the in the range of 25 – 30°C. Indirect sunlight was the energy source of *C. vulgaris* growth. These conditions were maintained throughout the duration of the experiment. Microalgae growth was determined by reading optical density at 680 wavelengths using Spectrophotometer (Fig. 3.4).
3.5.2 Phase 2: Examining the Effect of different cultural metabolism into the Growth of *C. vulgaris*, Nutrients Uptake, and Biomass Production

The objective of this stage was to study the effect of each cultural condition type (autotrophic, heterotrophic, mixotrophic condition) onto the growth of *C. vulgaris*, removal of nutrients, and production of biomass. The experiment consisted of 18 reactors (replicates), and it continued for 23 days. For autotrophic culture, atmospheric CO$_2$ was used as an inorganic carbon source for *C. vulgaris* growth. With respect to heterotrophic culture, 5 g/L of glucose, as an organic source of carbon, was injected and cultured under complete darkness using aluminum foil. In conjunction with mixotrophic culture, both organic and inorganic carbon sources were used, 2.5 g/L of glucose and atmospheric CO$_2$ respectively. For each reactor, *C. vulgaris* was initially added in a 10 percent ($V_{inoculated}/V_{of\ the\ medium}$) into Erlenmeyer flasks (500 ml) containing 400 ml of hydroponic and aquaponic wastewater samples. The experimental conditions were as following: initial pH adjusted between 7 to 9, temperature in the range of 25 – 30°C, a light intensity of 80 mol m$^{-2}$ s$^{-1}$, a light regime of 12:12 (light: dark) for mixotrophic conditions, and 24 hours of light regarding autotrophic conditions using white fluorescent lamps.
**3.5.3 Phase 3: Assessing the Effect of Different Nitrogen to Phosphorus (N:P) Molar Ratios into the Growth of *C. vulgaris*, Nutrients Uptake, and Biomass Production**

The objective of this phase was to examine the effect of N:P molar ratios into the growth of *C. vulgaris*, bioremediation of hydroponic and aquaponic wastewater samples, and production of biomass. Nine reactors (replicates) were used for 23 days. Cultures were grown into the modified Woods Hole MBL medium recipe with a change in NaNO$_3$ concentration in order to get the desired N:P molar ratios (8:1, 16:1, 24:1). Following to that, *C. vulgaris* was inculcated into the modified medium with the three different N:P molar ratios, 8:1, 16:1, 24:1. The tests were conducted at a temperature in the range of 25 – 30°C, pH was adjusted between 7-9 with light and dark cycle of 16 and 8 h using white fluorescent lamps.

**3.5.4 Phase 4: Examining the effect of non-sterilized Hydroponic and Aquaponic Wastewater under Mixotrophic Condition**

The objective of this phase was to study the effect of non-sterilization conditions with regard to hydroponic and aquaponic wastewater under mixotrophic conditions into the growth rate of *C. vulgaris*, removal of nutrients, and production of biomass. The experiment consisted of 18 reactors (replicates), 12 reactors including wastewater samples with microalgae and the others without microalgae serving as a control parameters. The experiment lasted for 23 days. Glucose was used as an organic carbon source corresponding to different initial concentrations of 5 g/L, 2.5 g/L while atmospheric CO$_2$ was used as inorganic carbon source. For each reactor, *C. vulgaris* was initially supplied in a 10 percent ($V_{inoculated}/V_{of\ the\ medium}$) in Erlenmeyer flasks (500 ml) containing 400 ml of the tested medium. The initial pH was adjusted between 7-9, temperature was in the in the range of 25 – 30°C, and light regime was 12:12 (light: dark) using white fluorescent lamps.
3.6 Analytical Methods

A variety of methods have been adopted for the experimental study as are mentioned in the following subsections.

3.6.1 Wastewater Quality Assay and Nutrient Analysis

Hydroponic and aquaponic wastewater are obtained from the effluent (disposal) tank located at the Center for Applied Research on the Environment and Sustainability (CARES) at the American University in Cairo, New Cairo, Egypt. The two sources of wastewater were analyzed for their physiochemical parameters, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), nutrients composition, total nitrogen (TN) and total phosphorous (TP), at the Agricultural Research Center at Cairo University. COD and BOD were measured based on the Standard Methods For the Examination of Water and Wastewater (Rice, Baird, & Eaton, 2017).

In conjunction with nutrients analysis, namely total nitrogen and total phosphorus, liquid samples of the culture media were collected at the end of the experimentations to determine total nitrogen (TN) and total phosphorus (TP) removals. In order to collect these samples, a predetermined volume of these samples were taken and subsequently filtered by a 0.45 Whatman GF/C membrane filter after the end of the each experiment. Following to that, total nitrogen was analyzed according to a method recommended by Jackson (1973) while total phosphorus was assessed based on a procedure described by APHA (1989). The removal efficiency of total nitrogen and total phosphorus is given by the Eq. (3.1):

\[
\text{Removal efficiency (\%)} = \left( \frac{x_0 - x_f}{x_0} \right) \times 100
\]  

(3.1)

Where \(x_0\) denotes the initial concentration of the nutrient while \(x_f\) signifies the final concentration of the nutrient.

3.6.2 Growth, Biomass, and Lipid Analysis

Microalgal growth were determined by measuring optical density (OD) at 680 nm using spectrophotometer (Fig. 3.6). Microalgal biomass was determined gravimetrically at initial, middle
and at the end of the experiments. A predetermined culture volume was harvested using sterilized filtrate and subsequently dewatered by centrifugation at 4000 rpm for 15 min. Following to that, a distilled water was used to wash and dry the microalgae C. vulgaris to a constant weight in the laboratory oven at 60 °C for the sake of dry weight biomass determination. The relationship between microalgae biomass concentration and optical density is given by the Eq. (3.2):

\[
\text{Dry weight (g/L)} = 0.628 \times \text{OD}_{680}
\]

The specific growth rates (\(\mu\)) and biomass productivity (\(P\)) are given by the Eq. (3.3) and the Eq. (3.4), respectively:

\[
\mu \ (\text{day}^{-1}) = (\ln N_2 - \ln N_1) / (t_2 - t_1)
\]

\[
P \ (\text{g/L/d}) = \frac{(N_2 - N_1)}{(t_2 - t_1)}
\]

Where \(N_1 \ (g/L)\) and \(N_2 \ (g/L)\) symbolize the biomass concentration at time \(t_1\) (day) and \(t_2\) (day) respectively. The previous equations have been used previously by Lam et al. (2017). By using these equations the growth rate and biomass productivity of C. vulgaris were calculated.

Total lipid content was performed according to a method reported by Bligh (1959) as follows: 8 milliliter (mL) of microalgae strain with 10 mL chloroform and 20 mL methanol were mixed and blended for 2 minutes using blender. This was done to achieve a balance of 1:2:0.8 (parts of chloroform: parts of methanol: water). The mixture then was again provided with another 10 mL chloroform, and it was blended for 30 seconds. In order to give a ratio of 2:2:1.8 (parts of chloroform: parts of methanol: water), 10 mL of distilled water was added and mixed for 30 seconds using blender. Whatman filter paper (No. 1) in line with Buchner funnel with slight section were used for the purpose of filtering the homogenate. The pressure was applied immediately with the bottom of a beaker when the residue dried. This was done in order to ensure the maximum recovery with respect to the solvent. Once the solvent was recovered, it was necessary to transfer the filtrate to a 50 mL graduated cylinder and to allow it for some minutes to complete the separation into two phases, then chloroform layer’s volume was recorded. A pipette was used to remove methanol phase with small volume of chloroform in order to ascertain a complete removal.
of methanol phase. Many pre-weighed dishes of aluminum were prepared, then they were provided with a 5 mL of the chloroform layer. In order to evaporate the chloroform, the aluminum dishes were heated in the fume hood. When the heating process completed, thin layers of lipid were left in the aluminum dishes, and immediately the dishes were dried in a drying oven at 105°C for 15 minutes in order to remove any remained chloroform’s traces. A desiccator was used for long time for cooling the aluminum dishes, and the dishes were weighted again to determine the lipid content. Subsequently, Eq. (3.5), which was used previously by Guldhe et al. (2017), was used to calculate the lipid productivity of C. vulgaris:

\[
\text{lipid productivity (g/L/d)} = \frac{\text{biomass productivity (g/L/d)}}{100} \times \frac{\text{lipid content}}{100} \quad (3.5)
\]
Chapter 4 RESULTS AND DISCUSSION

4.1 General

This chapter presents details regarding wastewater sampling collected from the effluent (disposal) tank located at the Center for Applied Research on the Environment and Sustainability (CARES) at the American University in Cairo, cultivation of C. vulgaris in the lab, experimental procedures, growth kinetics, findings of the research, focusing mainly on bioremediation of hydroponic and aquaponic wastewater in terms of nutrients removal and production of biomass, and their discussions.
4.2 Characteristics of Hydroponic and Aquaponic wastewater samples

The wastewater quality parameters of the hydroponic and aquaponic wastewater samples collected from the effluent (disposal) tank located at the Center for Applied Research on the Environment and Sustainability (CARES) at the American University in Cairo were assessed in terms of total nitrogen (TN), total phosphorus (TP), COD, and BOD as shown in Table 4.1. The two sources of wastewater used in this research were considered to be a promising source with regard to the growth of microalgae, the removal of nutrients, and production of biomass.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hydroponic Raw Wastewater</th>
<th>Aquaponic Raw Wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD (mg/L)</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>BOD (mg/L)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>TN (mg/L)</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>TP (mg/L)</td>
<td>32</td>
<td>1.35</td>
</tr>
</tbody>
</table>

4.3 *C. vulgaris* inoculum in the lab

*C. vulgaris* was inculcated in cylindrical glass bottles containing 400 ml of sterilized Woods Hole MBL medium recipe as recommended for freshwater microalgae at 22 (+/−3 °C (Fig. 4.1). Magnetic stirrer was used at 100 rpm to perform a regular mixing of the cultivation medium. The experiment (inculcation stage) was performed under stationary conditions in which pH of the medium was adjusted between 7-8 and light intensity of 80 mol m⁻² s⁻¹. The illumination was provided by fluorescent lamps under a light regime of 16/8 (light: dark cycle). After two weeks, a specified volume of microalgal culture was taken and inculcated in a new Woods Hole MBL medium. The microalgal culture was inculcated into hydroponic and aquaponic wastewater samples immediately after reaching a reasonable concentration.
4.4 Experimental Phase 1 (Initial Phase): Evaluation of *C. vulgaris* growth, Nutrients Uptake, and Biomass Production in Hydroponic and Aquaponic Wastewater Samples and Synthetic Media (MBL)

The objective of this phase was to examine to what degree *C. vulgaris* grows and removes nitrogen and phosphorous in hydroponic and aquaponic wastewater samples and to compare its growth in these samples with the standard growth medium for green algae (MBL). This experimental phase was fundamental as other experimental phases depended on its results so that based on the performance of *C. vulgaris* in hydroponic and aquaponic wastewater samples, microalgae strain was used to evaluate other important parameters that affect the growth rate, nutrient removal, and biomass production of *C. vulgaris*.

4.4.1 Growth rate, Biomass concentration, and Biomass Productivity

A regular monitoring of *C. vulgaris* growth with regard to hydroponic and aquaponic wastewater samples was performed in order to characterize the growth kinetics of microalgae strain (growth rate, biomass concentration, and biomass productivity). The profile of absorbance at 680 nm wavelength during the growth of *C. vulgaris* in conjunction with hydroponic and aquaponic wastewater samples as well as MBL, for MBL was used as a standard medium for microalgae growth, are shown in Fig. 4.2. As can be seen from Fig. 4.2, *C. vulgaris* growth in MBL medium
was slow during the first 8 days due to not only the small number of cell densities of the added microalgae but also the adaptation of the strain to the new environment. After day nine, the growth of microalgae turns to be exponential, then it entered a stationary phase after day 15 until the end of cultivation. What standing out in this figure also is that C. vulgaris was able to adapt to the new environment (hydroponic wastewater sample) very quickly and immediately entered the exponential phase, then a stationary phase took place from day 11 to day 16. After 17 days of cultivation, C. vulgaris growth started to decline gradually which was contrary to the growth patterns of the standard medium. With respect to the growth patterns of C. vulgaris in aquaponic wastewater sample, what can be clearly seen in Fig. 4.2 is that growth patterns in aquaponic wastewater sample was almost analogous to that of standard medium (MBL); however, C. vulgaris growth entered the exponential phase after day nine until reached the stationary phase after day 17, then started to decline. A possible explanation to the declining of C. vulgaris in hydroponic and aquaponic wastewater samples after stationary phase can be attributed to the reduction of the amount of nutrients in both samples. This reduction indicates that nutrients availability is one of the fundamental factors for enhancing the growth of microalgae.

Table 4.2 illustrates the kinetic parameters of the tested mediums. It can be seen from the data presented in Table 4.2 that maximum biomass concentration and biomass productivity were obtained for standard medium (MBL) that of 0.5 g/L and 0.03 g/L/d, respectively, then followed by 0.45 g/L and 0.026 g/L/d of biomass concentration and biomass productivity for aquaponic wastewater sample. On the other hand, biomass concentration and productivity were lower in hydroponic raw wastewater sample compared to that for aquaponic wastewater sample and MBL. As can be seen in the last column of the tables that the growth rate of microalgae in aquaponic wastewater sample and MBL were almost the same while the rate was smaller in hydroponic wastewater sample. The higher biomass concentration and biomass productivity obtained for the standard medium could be due to the remaining amounts of nutrients in the medium that might support the growth of microalgae and subsequently enhance the biomass productivity and biomass concentration. It is likely that the remaining amount of nutrients in hydroponic and aquaponic raw wastewater samples after the end of stationary phase was not enough to support the growth of C. vulgaris, and therefore affecting its growth. However, biomass concentration and biomass productivity were higher in aquaponic wastewater (lower initial nutrient concentration) sample.
compared with hydroponic wastewater sample which had the higher initial nutrient concentration. This indicates that insufficient nutrients in aquaponic wastewater sample did not prevent the growth of microalgae, and therefore resulting in higher growth rate compared with hydroponic wastewater sample. A possible explanation to this discrepancy might be the tendency of hydroponic wastewater sample to have lower pH values between 5-6 that could negatively affect the growth of *C. vulgaris*. On the other hand, aquaponic wastewater sample’s pH was in the range of 8-10.5 which is the best range for the growth of *C. vulgaris* (Rachlin & Grosso, 1991).

![Growth curve (absorbance, OD$_{680}$) of *C. vulgaris* during its acclimation in hydroponic and aquaponic wastewater samples as well as MBL](image)

**Figure 4.2:** Growth curve (absorbance, OD$_{680}$) of *C. vulgaris* during its acclimation in hydroponic and aquaponic wastewater samples as well as MBL.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Biomass Concentration (g/L)</th>
<th>Biomass Productivity (g/L/d)</th>
<th>Growth Rate (d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBL</td>
<td>0.5</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Hydroponic WW</td>
<td>0.38</td>
<td>0.021</td>
<td>0.07</td>
</tr>
<tr>
<td>Aquaponic WW</td>
<td>0.45</td>
<td>0.026</td>
<td>0.081</td>
</tr>
</tbody>
</table>

**Table 4.2:** kinetic parameters for microalga growth in of hydroponic wastewater, aquaponic wastewater, and MBL

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4.4.2 Nutrients Removal by *C. vulgaris* in Hydroponic and Aquaponic Wastewater Samples

According to the data presented in Table 4.3, maximum removal efficiency of nutrient was achieved in both aquaponic and hydroponic wastewater samples. Higher removal efficiency of total phosphorous (98%) was obtained in aquaponic wastewater sample followed by a removal efficiency of 90% for total nitrogen. In hydroponic raw wastewater sample, maximum total removal efficiency of phosphorus was also obtained (95%) while total nitrogen removal was 92%. These results indicate that the utilization of *C. vulgaris* for bioremediation of hydroponic and aquaponic wastewater samples is highly attainable. Microalgae have higher capacity to remove nutrients from wastewater as they use them for various physiological processes to generate biomass rich in lipids, carbohydrates, and proteins. Nitrogen is critical nutrient that is used by microalgae for protein as well as genetic material synthesis, also phosphorous is another important nutrient that is necessitated for short term storage and transfer of energy (Kim et al., 2016). This might explain the higher removal of total nitrogen and total phosphorous that were obtained in hydroponic and aquaponic wastewater samples, as microalgae use these nutrients for survival.

Based on the findings of this experimental phase, hydroponic and aquaponic wastewater samples were found to be a good medium for the growth of *C. vulgaris*, and the subsequent removal of nutrient as well as the production of biomass. Therefore, other experimental phases were conducted to evaluate various parameters in order to enhance the growth of *C. vulgaris*, to remove the nutrient, and to produce a reasonable amount of biomass.

<table>
<thead>
<tr>
<th>Raw wastewater sample</th>
<th>TN removal efficiency (%)</th>
<th>TP removal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquaponic wastewater</td>
<td>90</td>
<td>98</td>
</tr>
<tr>
<td>Hydroponic wastewater</td>
<td>92</td>
<td>95</td>
</tr>
</tbody>
</table>

*Table 4.3*: Total nitrogen and total phosphorous removal efficiency regarding hydroponic and aquaponic wastewater samples
4.5 Experiment Phase 2: Examining the Effect of different cultural metabolisms

The present experimental phase was designed to study the effect of each cultural condition type (autotrophic, heterotrophic, mixotrophic conditions) onto the growth rate of *C. vulgaris*, the removal of nutrients, and the production of biomass. Hydroponic and aquaponic wastewater samples were grown autotrophically, heterotrophically, and mixotrophically for 23 days in order to test the growth of *C. vulgaris*, to assess its ability to bioremediate wastewater samples, and to evaluate its biomass productivity. Fig. 4.3 shows *C. vulgaris* grown in hydroponic and aquaponic wastewater samples under different growth conditions.

![Cultivation of C. vulgaris](image)

*Figure 4.3: Cultivation of C. vulgaris in hydroponic and aquaponic wastewater samples under different growth conditions*

The inoculum of *C. vulgaris* was grown in aquaponic and hydroponic wastewater samples (Fig. 4.3) in order to get the amount of starter inoculum that can be used for bioremediation of wastewater samples and production of biomass. The profile of the absorbance at 680 nm wavelength during the acclimation of microalgae strain in hydroponic and aquaponic wastewater samples are shown in Fig. 4.4 and Fig. 4.5 respectively. As can be seen from Fig. 4.4 (aquaponic raw wastewater sample), the OD<sub>680</sub> values for the growth of *C. vulgaris* after 23 days of cultivation in mixotrophic, heterotrophic, and autotrophic were 1.075, 0.5, and 0.4 respectively, and microalgae growth in mixotrophic and heterotrophic was higher than that for autotrophic conditions. Growth rates of *C. vulgaris* were 0.13 d<sup>-1</sup> in mixotrophic, and 0.09 d<sup>-1</sup>, 0.08 d<sup>-1</sup> in heterophonic and autotrophic conditions respectively. With regard to hydroponic wastewater
sample, the OD_{680} values of *C. vulgaris* growth in mixotrophic, heterophonic, and autotrophic conditions after the end of cultivation were 1.3, 1.2, and 0.23 respectively, and the growth of microalgae was higher in mixotrophic and heterotrophic conditions compared to autotrophic conditions (Fig. 4.5). *C. vulgaris* growth rates in hydroponic wastewater sample in mixotrophic, heterotrophic, and autotrophic modes were 0.15 d^{-1}, 0.14 d^{-1}, and 0.07 d^{-1}, respectively.

The most fundamental factor in connection to the autotrophic growth of microalgae is light intensity and atmospheric CO$_2$, as microalgae use both of them as a major source for energy as well as carbon, correspondingly. On the other hand, microalgae use organic carbon as the sole source with respect to carbon and energy for heterotrophic conditions which directly affects this type of cultural metabolism (Cheirsilp & Torpee, 2012). Moreover, mixotrophic microalgae use light as energy source and both organic and inorganic carbon as carbon source. A possible explanation for the slower growth rate with regard to autotrophic conditions observed in this phase of experiment might be because of photoinhibition owning to the high microalgal cells and the shading phenomena. This effect indicates that microalgal cells did not receive enough light due to the high density of microalgal cells that were present in the upper zone of the culture (light did not distributed equally) or due the low ambient atmospheric CO$_2$ concentration (Bux & Chisti, 2016; Larsdotter, 2006). However, *C. vulgaris* growth in both mixotrophic and heterotrophic cultures were higher indicating that organic carbon and light were the major reasons for the higher growth of the strain.

Previous studies assessing the effect of different cultural metabolisms have shown that microalgae growth under heterotrophic is higher than that for autotrophic conditions (Liang, Sarkany, & Cui, 2009; Shi, Zhang, & Chen, 2000; Zheng, Chi, Lucker, & Chen, 2012). Several reports have shown that microalgal cultivation under mixotrophic condition is higher than that for both autotrophic and heterotrophic conditions. For instance, (Yu, Shi, Cai, Cong, & Ouyang, 2011) examined the growth of different cultural conditions into the growth of *C. globose* and the observation was that the growth of microalgae under mixotrophic conditions was higher in comparison with heterophonic and autotrophic conditions. In another study also conducted by Bhatnagar et al. (2011) to assess the effect of mixotrophic, heterotrophic, and autotrophic conditions onto the growth of *C. minutissima*, they confirmed that the growth rate of microalgae
under mixotrophic conditions was more than 2 times than that for heterophonic conditions and 7 times than that for autotrophic conditions.

Considering the fact that mixotrophic microalgae undergo photosynthesis, and both organic and inorganic (CO$_2$) compounds are used as a carbon source for the microalgal growth. This might be the major reason for the stimulated growth of *C. vulgaris* under mixotrophic culture. (Chojnacka & Noworyta, 2004) investigated the growth of Spirulina sp. under photoautotrophic, heterotrophic and mixotrophic cultures, and they reported that the specific growth rate in mixotrophic conditions with light intensity of 33Wm$^{-2}$ was higher than for heterotrophic and autotrophic cultures. On the contrary, the specific growth rate under heterophonic culturing of microalgae with light intensity of 17 Wm$^{-2}$ was higher than that for mixotrophic conditions. These results indicate that light intensity is a critical factor in connection to mixotrophic culturing of microalgae which directly affects the growth of microalgae and subsequently slower the specific growth rate under mixotrophic conditions compared to heterophonic conditions.

Comparing hydroponic and aquaponic wastewater samples growth under mixotrophic, heterotrophic and phototrophic conditions, as can be seen in Fig. 4.6, Fig. 4.7, and Fig. 4.8, microalgal growth under mixotrophic and heterotrophic cultures was higher in hydroponic wastewater sample than that for aquaponic wastewater sample. These observations indicate that the composition of nutrients in hydroponic wastewater enhanced the growth of microalgae resulting in higher growth rate compared to aquaponic wastewater sample. On the other hand, insufficient nutrients in aquaponic wastewater sample did not adversely affect the growth of microalgae under mixotrophic conditions which might be probably due to both supplemented organic carbon and atmospheric CO$_2$. In addition, this was not the case in heterophonic conditions when organic source was the sole source for carbon as well as energy as can be observed in Fig. 4.7. This results are likely to be related to the lower nutrients composition of aquaponic wastewater sample.

Contrary to mixotrophic and heterotrophic growth profiles, microalgae growth under autotrophic culture was higher in aquaponic wastewater sample compared to hydroponic wastewater sample (Fig. 4.8). This indicates that insufficient nutrients in aquaponic wastewater
sample did not prevent the growth of microalgae under autotrophic culture, and therefore resulted in higher growth rate compared with hydroponic wastewater. A possible explanation to this discrepancy might be because of the tendency of hydroponic wastewater observed under autotrophic during the experiment towards lower pH values between 5-6 that might affect the growth of *C. vulgaris*. On the other hand, aquaponic wastewater sample’s pH was in the range of 8-10.5 which is the best range for the growth of *C. vulgaris* (Rachlin & Grosso, 1991).

**Figure 4.4**: Growth curve (absorbance, OD₆₈₀) of *C. vulgaris* during its acclimation aquaponic wastewater samples as under different growth conditions.
Figure 4.5: Growth curve (absorbance, OD$_{680}$) of C. vulgaris during its acclimation in hydroponic wastewater sample as under different growth conditions.

Figure 4.6: Growth curve (absorbance, OD$_{680}$) of C. vulgaris during its acclimation in hydroponic and aquaponic wastewater samples as under mixotrophic growth conditions.
4.5.1 Algal Nutrients Removal of Hydroponic and Aquaponic Wastewater Samples

The total nitrogen removal amounts with regard to hydroponic wastewater sample were 27 mg/L, 28.8 mg/L, 29.55 mg/L in autotrophic, heterotrophic, and mixotrophic conditions, respectively, and the removal amount in mixotrophic conditions was higher than that for autotrophic and heterotrophic cultures. Phosphorus was nearly removed completely in all cultural conditions. Total
nitrogen and phosphorus removal efficiencies were 98.5% and 99.99% in mixotrophic conditions, 96% and 99.99% in heterophonic conditions, and 90% and 91.3% in autotrophic conditions. In connection to aquaponic wastewater sample, total nitrogen removal amounts was 9.89 mg/L in mixotrophic condition, 9.78 mg/L in heterotrophic condition, and 9.4 mg/L in autotrophic conditions while total phosphorus removal amount was 1.34 mg/L, 1.3477 mg/L, 1.35 mg/L in autotrophic, heterotrophic, and mixotrophic conditions, respectively. Moreover, total nitrogen removal efficiency for aquaponic wastewater sample was 94%, 97.8%, 98.5% in autotrophic, heterotrophic, and mixotrophic conditions, respectively, and the total removal efficiency of phosphorus was 99%, 99.9%, 99.93% in autotrophic, heterotrophic, and mixotrophic conditions, respectively.

Microalga have higher capacity to remove nutrients from wastewater as they use them for various physiological processes to generate biomass rich in lipids, carbohydrates, and proteins. Nitrogen is critical nutrient that is used by microalgae for protein as well as genetic material synthesis, also phosphorous is another important nutrient that is necessitated for short term storage and transfer of energy (Kim et al., 2016). Inorganic nitrogen compounds (ammonium, nitrate and nitrite) are utilized by microalgae through a process of assimilation in which nitrite and nitrate undertake reduction to form ammonium which is subsequently combined in amino acid glutamine (Cai et al., 2013b). Removal efficiency of total nitrogen from hydroponic and aquaponic wastewater samples under mixotrophic and heterotrophic growth was higher than that for autotrophic growth of C. vulgaris while total phosphorus removal efficiency where almost same in all cultural metabolisms, expect that for autotrophic conditions in hydroponic wastewater.

During heterotrophic mode of microalgae, the organic carbon source presence in the medium plays an essential role regarding the removal efficiency of both nitrogen and phosphorus. On the other hand, microalgae use light and both organic and inorganic carbon source during mixotrophic growth to enhance the microalgal growth and therefore increasing the removal efficiency of nutrients. The addition of glucose as a carbon source for both heterotrophic and mixotrophic might be a major reason for the higher nitrogen and phosphorous removal efficiency that were obtained from this experimental phase compared of that for autotrophic growth. According to Martinez et al. (2000), the transfer rate of energy absorbed by microalgae with regard
to ATP for autotrophic, heterotrophic, and mixotrophic are 10%, 18%, and 12% respectively. In connection to autotrophic growth, ATP is generated from mitochondria then around 77% of ATP is used for fixation of CO$_2$ via Calvin cycle while the remaining is converted to organic compounds. According to that explanation, the small growth rate and removal efficiency of nitrogen and phosphorous of autotrophic mode can be attributed to low energy portion for cell synthesis in comparison with heterotrophic and mixotrophic modes.

However, the higher removal efficiency of nitrogen and phosphorous obtained from this experimental phase under autotrophic conditions (around 90%) indicates that the utilization of *C. vulgaris* for bioremediation of hydroponic and aquaponic wastewater samples is also a plausible strategy for removing nitrogen and phosphorous from wastewater. On the other hand, almost complete removal of nitrogen and phosphorus has been achieved under mixotrophic and heterotrophic culturing of *C. vulgaris* for hydroponic and aquaponic wastewater samples which might be possibly due to the higher transfer rate of energy absorbed by microalgae with regard to ATP under these modes. These findings are in consistent with other results obtained from literature. In a study conducted by Wang & Lan, (2011) to examine the removal efficiency of nutrients by using Neochloris oleoabundans cultured in a synthetic secondary municipal wastewater effluent, they found that total nitrogen removal can reach 99% based on nitrogen to phosphorous ratio while a complete removal of phosphorous was achieved. Almost 100% removal efficiencies for total nitrogen and total phosphorous were obtained by Yang et al. (2016). This higher removal efficiencies were corresponding to the initial total nitrogen and total phosphorous of 12 mg/L and 1.8 g/, respectively. In another study conducted by L. Wang et al. (2010) to assess the ability of green algae Chlorella sp. for the sake of nutrients removal from the effluent of the primary treatment unit, total nitrogen and phosphorous removal were 68.5% and 90.6% respectively. Zheng et al. (2012) reported that under autotrophic conditions of Chlorella sp., the removal rates of nitrogen and phosphorous were 74.7% and 90.6%, respectively. L. Wang et al. (2010) and Zheng et al. (2012) results for total nitrogen removal were lower than the results obtained from this study which might be likely due to initial total nitrogen and phosphorous concentrations, N:P molar ratio, and other characteristics of the used wastewater samples in these studies. Overall, mixotrophic and heterotrophic culturing of microalgae seems to be the most
effective types of cultural metabolisms with regard to bioremediation of hydroponic and aquaponic wastewater samples used in this research work.

4.5.2 Biomass Concentration and Biomass Productivity of C. vulgaris

Biomass concentration (g/L) and biomass productivity (g/L/d) under mixotrophic, heterotrophic, and autotrophic culturing of microalgae in hydroponic and aquaponic wastewater samples are shown in Fig. 4.9 and Fig. 4.10. In hydroponic wastewater sample, biomass concentration and biomass productivity under mixotrophic growth were 1.26 g/L and 0.1108 g/L/d which were higher than that of heterotrophic growth (1.03 g/L and 0.107 g/L/d) and autotrophic growth (0.23 g/L and 0.016 g/L/d). In aquaponic wastewater sample, biomass concentration and biomass productivity were also higher under mixotrophic condition (0.99 g/L and 0.089 g/L/d) while under heterotrophic and autotrophic growth were 0.55 g/L, 0.036 g/L/d and 0.33 g/L, 0.032 g/L/d respectively. Furthermore, biomass concentration and productivity were higher in hydroponic wastewater sample compared to aquaponic wastewater sample under both mixotrophic and heterotrophic growth (Fig. 4.12 and Fig. 4.13). On the other hand, biomass concentration and productivity were higher in aquaponic wastewater compared with hydroponic wastewater under autotrophic growth (Fig. 4.11).

The lower biomass concentration and biomass productivity obtained during autotrophic growth compared with heterotrophic and mixotrophic growth in both hydroponic and aquaponic wastewater samples might be due to photoinhibition that indicates light deficiency as some microalgal cell do not receive enough light because of shading phenomena (Bux & Chisti, 2016; Larsdotter, 2006). In addition, light energy, the amount of light received by microalgae, that is directly correlated with photon flux density that hits the surface of the culture also might be a possible reason for the lower biomass concentration and productivity as it directly affects microalgal growth and therefore biomass productivity (Richmond, 2004). Furthermore, insufficient inorganic carbon supplementation might be also a probable cause for the lower biomass concentration and biomass productivity due to the low ambient atmospheric concentration. This observation supports evidence from previous studies. For example, Abedini Najafabadi et al. (2015) reported a biomass concentration and biomass productivity of 1.1 g/L and 0.076 g/L/d when no carbon were supplied (with fresh air) while cell density and biomass
productivity were increased when carbon was supplemented with 3% CO₂ resulting in 1.46 g/L of biomass concentration and 0.111 g/L/d of biomass productivity.

Under mixotrophic culturing of microalgae, there are two distinctive processes, photosynthesis and aerobic respiration which means that microalgae use light as energy source while they use both organic and inorganic carbon as carbon source. The highest biomass concentration and productivity were obtained during mixotrophic growth of *C. vulgaris* with 2.5 g/L glucose followed by heterotrophic growth in both hydroponic and aquaponic wastewater samples. Some studies have demonstrated that *C. vulgaris* possesses a hexose transport system that can be achieved by glucose (Tanner 1969; Komor and Tanner 1971; Haass and Tanner 1974). The current research found that *C. vulgaris* can grow well on hydroponic and aquaponic wastewater sample supplemented with a predetermined glucose concentration under mixotrophic and heterotrophic conditions. This carbon source addition has resulted in higher biomass concentration and biomass productivity of *C. vulgaris* during mixotrophic and heterotrophic growth compared with autotrophic growth. In addition, *C. vulgaris* can be affected also by the light as reported in some studies that some strains of *C. vulgaris* grow only in darkness (Haass and Tanner 1974) while others grow only under the presence of light (Karlander and Krauss 1966). Based on this experimental phase observations, it can be concluded that *C. vulgaris* grows better under mixotrophic growth and therefore resulting in higher biomass concentration and biomass productivity compared to heterotrophic and autotrophic culture. This result have been supported by other studies. For instance, Andruleviciute et al. (2014) reported that the highest biomass concentration and productivity can be obtained with the supplementation of organic carbon source for Chlorella sp., Scenedesmus sp., Haematococcus sp. and Nannochloris sp., respectively, under mixotrophic conditions. Liang et al. (2009) reported that biomass concentration under mixotrophic growth has been enhanced from 0.25 g/L under autotrophic conditions to 0.77 g/L when glycerol was added as a carbon source.

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Figure 4.9: Biomass concentration (g/L) and biomass productivity (g/L/d) under mixotrophic, heterotrophic, and autotrophic conditions in hydroponic wastewater sample.

Figure 4.10: Biomass concentration (g/L) and biomass productivity (g/L/d) under mixotrophic, heterotrophic, and autotrophic conditions in aquaponic wastewater sample.
Figure 4.11: Biomass concentration (g/L) and biomass productivity (g/L/d) under autotrophic conditions in hydroponic and aquaponic wastewater sample

Figure 4.12: Biomass concentration (g/L) and biomass productivity (g/L/d) under heterotrophic conditions in hydroponic and aquaponic wastewater sample
4.5.3 Lipid Accumulation of C. vulgaris under Autotrophic, Heterotrophic, and Mixotrophic Conditions

As shown in Fig. 4.14, C. vulgaris produced its highest lipid content values under heterotrophic, followed by mixotrophic and autotrophic conditions. The final lipid content was 0.374 g/L (29.8 wt% on an ash-free dry weight “AFDW”), 0.341 g/L (33 wt% AFDW), and 0.0342 g/L (15 wt% AFDW) under mixotrophic, heterotrophic, and autotrophic conditions, respectively in hydroponic wastewater. In aquaponic wastewater, the final lipid content was 0.239 g/L (24.2 wt% AFDW) under mixotrophic growth, 0.206 g/L (37 wt% AFDW) under heterotrophic growth, and 0.063 g/L (19 wt% AFDW) under autotrophic growth (Table 4.4). It appears that the highest lipid production was obtained under mixotrophic growth followed by heterotrophic growth and autotrophic growth while the highest lipid productivity, which is a product of biomass productivity and lipid content, was obtained under heterotrophic conditions in hydroponic wastewater sample and under mixotrophic conditions in aquaponic wastewater sample (Table 4.4).

Lipid is one of the pivotal outcomes in conjunction with microalgal biomass production, and it produces by microalgae for the purpose of energy saving regarding their cytoplasm. Lipid also is a key factor for the production of biofuels through various processes, such as esterification. The highest lipid concentration was obtained under mixotrophic conditions in both hydroponic
and aquaponic wastewater samples compared to that accumulated under heterotrophic and autotrophic modes in *C. vulgaris* cells, even though, the lipid content was higher under heterotrophic mode. The higher biomass production obtained under mixotrophic conditions might be a possible reason for the highest lipid concentration, noted that lipid concentration is a product of dry weight and lipid content. The distinctive process regarding mixotrophic microalgae which use both organic and inorganic carbon as a carbon source played a vital role to enhance the biomass production of microalgae and subsequently the lipid producing ability of *C. vulgaris*. The two sources of carbon, glucose and atmospheric CO₂ were used in this research work in order to grow *C. vulgaris* mixotrophically indicating that carbon was available for the growth of *C. vulgaris*, and therefore boosted the proportion of the storage lipids. Heterotrophic also is a plausible strategy for lipid production, and it is considered to of benefit in connection to lipid accumulation which is obvious based on the highest lipid content obtained under this mode (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011b). It also seems possible that the presence of organic carbon (glucose) as the sole source of carbon and energy for heterotrophic growth enhanced the lipid content of *C. vulgaris*; however, the lowest lipid concentration obtained under this mode compared with mixotrophic mode might be due to some factors, such as the glucose concentration and nitrogen concentration (Mohammad Mirzaie, Kalbasi, Mousavi, & Ghobadian, 2016). While autotrophic microalgae use light and inorganic carbon as both energy and carbon sources, the obtained lipid concentration and lipid content were very small compared to that for mixotrophic and heterotrophic growth. Low ambient atmospheric CO₂ and unequal light distribution among microalgal cells might be a probable cause that affected the lipid producing ability of *C. vulgaris* under autotrophic cultivation.

In accordance with the present results, previous studies have demonstrated that highest lipid production and lipid content can be obtained under mixotrophic and heterotrophic modes of cultivation. In a study conducted by Mohammad et al. (2016), a maximum lipid concentration of 0.86 g/L was obtained under mixotrophic conditions corresponding to maximum biomass concentration of 2.62 g/L. Liu et al. (2011) reported a higher lipid content of *C. protothecoides* under heterotrophic mode in comparison with that for photoautotrophic mode. Liang et al. (2009) reported a higher lipid content of *C. vulgaris* cultivated under photoautotrophic conditions compared to that for mixotrophic and heterotrophic cultivations which is not in accord with the
results presented in the present research work. This rather contradictory result may be due to enhanced light intensity and inorganic source of carbon supplementation, for the light intensity and inorganic carbon source are the fundamental factors for enhancing the biomass production as well as the lipid producing ability of microalgae. It is possible, therefore, that the lipid producing ability of microalgae is dependent on many factors, such as strain selection, organic and inorganic carbon source, and light intensity. However, it is obvious that the addition of glucose as an organic source of carbon played an essential role for enhancing both the biomass and the lipid producing ability under both mixotrophic and heterotrophic growth in the current study. Despite these promising results, it is of paramount importance to further investigate mixotrophic, heterotrophic, and autotrophic cultivations under different conditions, particularly regarding the effect of different sources of carbon and the effect of light intensity in future research. Such examination would play an important role for sustainable production of biofuels and simultaneously boosting the prospect of large cultivation facilities of biorefineries.

Figure 4.14: C. vulgaris lipid content under mixotrophic, heterotrophic, and autotrophic conditions in hydroponic and aquaponic wastewater samples
### Table 4.4: Lipid production and lipid productivities under different growth modes in hydroponic and aquaponic wastewater samples

<table>
<thead>
<tr>
<th>Growth Mode</th>
<th>Hydroponic wastewater</th>
<th>Aquaponic wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipid production (g/L)</td>
<td>Lipid Productivity (g/L/d)</td>
</tr>
<tr>
<td>Autotrophic growth</td>
<td>0.0342</td>
<td>0.0024</td>
</tr>
<tr>
<td>Heterotrophic growth</td>
<td>0.341</td>
<td>0.0354</td>
</tr>
<tr>
<td>Mixotrophic growth</td>
<td>0.374</td>
<td>0.033</td>
</tr>
</tbody>
</table>

### 4.6 Experimental Phase 3: Assessing the Effect of Different Nitrogen to Phosphorus (N:P) Molar Ratios into the Growth of C. vulgaris, Nutrients Uptake, and Biomass Production

This phase of research set out with the aim of assessing the effect of N:P molar ratios into the growth of *C. vulgaris*, removal of nutrients in hydroponic and aquaponic wastewater samples, and subsequent production of biomass. Fig. 4.15 shows *C. vulgaris* grown in MBL with different N:P molar ratios.

![Figure 4.15: Cultivation of C. vulgaris in MBL with different N:P molar ratios: (A) after twelve days of cultivation (B) by the end of cultivation](image-url)
4.6.1 Growth rate, Biomass concentration, and Biomass Productivity

Growth conditions for *C. vulgaris* were optimized for the purpose of enhancing the growth of microalgae strain into a modified Woods Hole MBL medium recipe solution, of enhancing the removal efficiency of nutrients, and of producing a considerable amount of biomass. The regular monitoring of microalgal growth into different cultures allowed the characterization of *C. vulgaris* growth kinetics with different N:P molar ratios. The profile of absorbance at 680 nm wavelength during the growth of *C. vulgaris* in conjunction with the tested mediums are shown in Fig. 4.16. In general, the growth of *C. vulgaris* under different nitrogen to phosphorous ratios presented the same growth behavior as can be seen in Fig. 4.16 that adaptation phase was for three days while exponential phase started from the day five of culture until the end of cultivation.

Fig. 4.17 presents the major kinetic parameters (specific growth rate, biomass concentration, and biomass productivity). With regard to the specific growth rate, the maximum value was obtained for N:P ratio of 8:1 which was 0.08 day\(^{-1}\), and values of 0.075 day\(^{-1}\) and 0.073 day\(^{-1}\) were obtained for N:P molar ratios of 16:1, and 24:1, respectively. On the other hand, maximum biomass concentrations of 0.204 g/L was obtained for N:P ratio of 8:1, followed by 0.184 g/L for N:P ratio of 16:1, and 0.164 g/L for N:P ratio of 24:1. Biomass productivity was almost the same in all molar ratios. A possible explanation for the higher biomass concentration and growth rate obtained for N:P ratio of 8:1 might be because of nitrogen limitation as microalgae achieve higher biomass and lipid productivities under nitrogen starvation conditions (C. Y. Chen et al., 2011).

These results are in agreement with other studies found in the literature. (Silva et al., 2015) estimated the effect of different N:P ratios (8:1, 16:1, and 24:1) into the growth of microalgae *C. vulgaris* and *P. subcapitata*. The most preferred N:P molar ratio for the growth of microalgae *C. vulgaris* was found to be 8:1. Hadj-Romdhane et al. (2012) examined the effect of different nitrogen to phosphorous molar ratios onto the growth of *C. vulgaris*, and they concluded that a reasonable growth could be reached near 8:1. In another important study conducted by Christopher A. Klausmeier et al. (2004) to assess the influence of N:P molar ratios into the growth of microalgae, the study indicated that the values can vary from 8.2 to 45 according to the experimental conditions. The study also suggested that Redfield ratio is an average of the values
achieved regarding the various species. Nevertheless, cultivation of microalgae should be done in continuous mode during the exponential growth phase that would lead to higher biomass concentration and subsequently higher biomass productivity. It is also paramount that enhancing the growth of microalgae should be accomplished under the optimal cultural conditions such as that of optimal N:P molar ratio which gives a higher biomass concentration. Based on this experimental phase, *C. vulgaris* achieved the maximum values for biomass concentration and growth rate for N:P molar ratio of 8:1, and therefore indicating that maximum biomass production could be possibly achievable under this ratio.

**Figure 4.16:** Growth curve (absorbance, OD$_{680}$) of *C. vulgaris* during its acclimation MBL with different N:P molar ratios
Concerning the effect of N:P molar ratios on nutrient removal by *C. vulgaris*, maximum removal efficiency of total nitrogen and total phosphorous was achieved for N:P ratio of 8:1 as shown in Table 4.5. It appears that maximum amount of phosphorous could be removed (88%) for N:P ratio of 8:1 followed by removal efficiency of 85% and 70% for N:P ratios of 16:1 and 24:1, respectively, based on this research work conditions. In addition, higher removal efficiency of nitrogen was achieved for 8:1 N:P molar ratio followed by 16:1 N:P ratio and 24:1 N:P ratio. It is obvious that nitrogen and phosphorous removal efficiency decreased somehow when nitrogen concentration increased, so that at N:P molar ratios of 16:1 and 24:1 a reasonable amounts of nutrients could not be removed effectively. This indicates that higher removal efficiency of nitrogen can be achieved when phosphorous is limited. Thus, N:P molar ratio of 8:1 presented higher removal efficiency in terms of nitrogen and phosphorus.

One of the fundamental factors with respect to the optimum nutrient ratio in wastewater is the composition of algal cells that can provide a good explanation for nutrient removal efficiency in wastewater. According to Stumm empirical formula for microalgae, the nitrogen to phosphorous molar ratio is 7.2:1; nevertheless, this is not the case in all conditions based on the fact that microalgal cells composition are dependent on the type of microalgal strain as well as growth conditions (Xin et al., 2010). Kapdan and Aslan (2008) studied the effect of different N:P molar
ratios on the removal efficiency of nutrient for *C. vulgaris*, and they found that the optimum removal efficiency was for N:P ratio of 8:1. This result supports the current research finding of that the optimum N:P ratio of 8:1 found to give higher removal efficiency in terms of nitrogen and phosphorous. In addition, it appears that the uptake of phosphorous and nitrogen by *C. vulgaris* followed the N:P ratio from the empirical formula for microalgae somehow. This suggests that higher removal of nutrient can be achieved under lower N:P molar ratios while higher ratios affect the removal of nutrient. Therefore, *C. vulgaris* was able to remove nitrogen and phosphorus under N:P molar ratio of 8:1 than that for higher molar ratios of 16:1 and 24:1. This finding was supported by other results found in the literature. For instance, Xin et al. (2010) studied the effect of various nitrogen and phosphorous concentration into the growth of Scenedesmus sp., and they found that higher removal efficiency of nitrogen (83-99%) and phosphorous (99%) were achieved for N:P molar ratio in the range of 5:1-12:1. Overall, it seems obvious that maximum removal of nutrient can be achieved under lower N:P ratios based on the findings of this research work as well as literature.

Table 4.5: Total nitrogen and total phosphorous removal efficiency for different N:P molar ratios

<table>
<thead>
<tr>
<th>N:P Molar Ratios</th>
<th>TN removal efficiency (%)</th>
<th>TP removal efficiency (%)</th>
</tr>
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<tbody>
<tr>
<td>8:1</td>
<td>85</td>
<td>88</td>
</tr>
<tr>
<td>16:1</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>24:1</td>
<td>75</td>
<td>70</td>
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</table>
4.7 Phase 4: Examining the effect of non-sterilized hydroponic and aquaponic wastewater under mixotrophic conditions

The objective of this phase was to evaluate the effect of non-sterilization conditions in connection to hydroponic and aquaponic raw wastewater samples under mixotrophic conditions into the growth rate of *C. vulgaris*, removal of nutrients, and production of biomass.

4.7.1 Growth rate, Biomass concentration, and Biomass Productivity

The profiles of absorbance at 680 nm wavelength for *C. vulgaris* growth in aquaponic and hydroponic wastewater samples are shown in Fig. 4.18 and Fig. 4.19. As can be seen from Fig. 4.18, *C. vulgaris* growth patterns regarding both initial glucose concentration of 5 and 2.5 g/L were very similar at the beginning, and they entered the exponential phase from second day till day five in aquaponic wastewater sample. After day six, *C. vulgaris* growth started to fluctuate for the initial glucose concentration of 2.5 g/L while the growth continued to increase for the initial glucose concentration of 5 g/L until day 15, then it started to decrease. On the other hand, no marked growth has been observed regarding the controlled conditions (aquaponic wastewater without microalgae) until day 18 when microalgae growth started to increase slightly. In conjunction with the growth of *C. vulgaris* in hydroponic wastewater sample, what can be seen clearly in Fig. 4.19 is the rapid growth of microalgae with respect to the initial glucose concentration of 2.5 g/L while the growth regarding that of 5 g/L was high during the first days of cultivation, then it started to fluctuate till the end of cultivation. No marked microalgae growth was observed with regard to the controlled conditions (hydroponic wastewater without microalgae).

The high microalgae growth regarding 2.5 g/L glucose in hydroponic wastewater sample might be due to both glucose, atmospheric CO\textsubscript{2}, and light. In specific, initial glucose concentration of 2.5 g/L was enough for microalgae to grow during night time unlike that for 5 g/L in which the growth of *C. vulgaris* was lower indicating that the amount of glucose was too much for microalgae to grow during night time. Another possible reason for the lower growth observed for 5 g/L glucose is the effect of non-sterilization meaning that competition of *C. vulgaris* with other microorganisms could be highly probable. On the contrary, the growth was higher for 5 g/L glucose in aquaponic wastewater sample. It seems possible that contradictory is due to low organic compounds of aquaponic wastewater sample compared to that for hydroponic wastewater sample.
Thus, the addition of 5 g/L glucose to the cultivation medium enhanced the organic load in the media and the growth of microalgae unlike that for 2.5 g/L indicating that the glucose was not enough for enhancing the growth of microalgae during night time. While *C. vulgaris* showed a reasonable growth patterns in both hydroponic and aquaponic wastewater samples, no marked growth regarding the controlled conditions (hydroponic and aquaponic wastewater samples without algae) has been observed. This indicates that *C. vulgaris* was able to grow in non-sterilized conditions without being affected significantly by other microorganisms.

Table 4.6 and Table 4.7 present the kinetic parameters with regard to microalgae growth in aquaponic and hydroponic wastewater samples, respectively. It can be seen from Table 4.6 that maximum biomass concentration (0.35 g/L) and biomass productivity (0.02 g/L/d) were obtained for the initial glucose concentration of 5 g/L with a growth rate of 0.08 d⁻¹ compared to 5 g/L glucose in aquaponic wastewater sample. With respect to the kinetic parameters in hydroponic wastewater sample, what can be seen in Table 4.7 is that higher growth rate (0.14 d⁻¹), biomass concentration (0.54 g/L), and biomass productivity (0.056 g/L/d) were attributed to cultivation medium supplied with 2.5 g/L glucose.

In mixotrophic growth, microalgae grow heterotrophically and autotrophically which play an important role for enhancing the kinetic parameters of microalgae. In another words, microalgae use light and both organic and inorganic source of carbon to grow which might indicate that a reasonable amount of biomass concentration and biomass productivity can be obtained under such growth conditions. The addition of organic source of carbon, such as glucose, is one of critical factors which enhances the growth parameters of microalgae. Glucose is the most preferred source of carbon regarding heterotrophic microalgae, and it plays a simultaneous role as a carbon source as well as energy source for enhancing the growth of heterotrophic microalgae. In addition, glucose affects the metabolic carbon assimilation in connection to *C. vulgaris* beside the size of the cells, the quantity of storage materials like lipids and protein, and the cellular contents of chlorophyll, RNA, and vitamins. The addition of glucose as a carbon source to hydroponic and aquaponic wastewater enhanced the concentration of carbon in these samples, and simultaneously enhanced the biomass concentration and biomass productivity of *C. vulgaris* (Morales-Sánchez, Tinoco-Valencia, Kyndt, & Martinez, 2013). However, different glucose concentrations affect the growth
rate, biomass productivity, and biomass concentration of microalgae according to the composition of the cultivation medium. The higher biomass concentration and productivity observed for hydroponic wastewater supplied with 2.5 g/l glucose indicates that 2.5 g/L was enough during the night time under mixotrophic growth in addition to atmospheric CO₂ as well as light. The lower biomass concentration and productivity observed for 5 g/L glucose might be due to the effect of non-sterilization. This means that when glucose was supplied with relatively high concentration, the organic load was increased in the cultivation media and subsequently other microorganisms competed with microalgae to assimilate the source, and therefore affecting the growth of C. vulgaris. In general, it is clear from this phase that C. vulgaris can grow mixotrophically under non-sterilized conditions. However, the observed growth patterns of C. vulgaris was not enough for achieving higher biomass production suitable for commercial application maybe due to the effect of non-sterilization. Therefore, more investigations regarding such conditions are needed in order to enhance the biomass production and simultaneously large cultivation facilities.

Figure 4.18: Growth curve (absorbance, OD₆₈₀) of C. vulgaris during its acclimation in non-sterilized aquaponic wastewater sample under different initial glucose concentrations
Figure 4.19: Growth curve (absorbance, OD$_{680}$) of C. vulgaris during its acclimation in non-sterilized hydroponic wastewater sample under different initial glucose concentrations.

Table 4.6: kinetic parameters for microalgae growth in non-sterilized aquaponic wastewater sample under different initial glucose concentration

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth rate (d$^{-1}$)</th>
<th>Biomass Concentration (g/L)</th>
<th>Biomass Productivity (g/L/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With 5 g/L Glucose</td>
<td>0.08</td>
<td>0.35</td>
<td>0.02</td>
</tr>
<tr>
<td>With 2.5 g/L Glucose</td>
<td>0.07</td>
<td>0.29</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Table 4.7: kinetic parameters for microalgae growth in non-sterilized hydroponic wastewater sample under different initial glucose concentration

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth rate (d$^{-1}$)</th>
<th>Biomass Concentration (g/L)</th>
<th>Biomass Productivity (g/L/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With 5 g/L Glucose</td>
<td>0.09</td>
<td>0.25</td>
<td>0.022</td>
</tr>
<tr>
<td>With 2.5 g/L Glucose</td>
<td>0.14</td>
<td>0.54</td>
<td>0.056</td>
</tr>
</tbody>
</table>
4.7.2 Nutrients Removals

Maximum removal efficiency with regard to total nitrogen (TN) and total phosphorous (TP) by microalgae were obtained for hydroponic wastewater sample supplied with initial glucose concentration of 2.5 g/L, 73.33% and 86.56%, respectively. On the other hand, TN removal efficiency of 53.3% and TP removal efficiency of 61.6% were obtained for hydroponic wastewater supplied with initial glucose of 5g/L. Furthermore, maximum treatment efficiency of 72% TN and 77.78% TP in conjunction with aquaponic wastewater sample was obtained for the initial glucose concentration of 5 g/L while the treatment efficiency of TN and TP regarding the initial glucose concentration of 2.5 g/L were 60% and 68.89%, respectively.

Under mixotrophic growth, microalgae use both inorganic and organic carbon as a carbon source, and they use light as energy source, which plays an important role for enhancing the treatment efficiency of nutrients from wastewater. The good treatment efficiency obtained with hydroponic wastewater supplied with initial glucose concentration of 2.5 g/L compared with that of 5 g/L suggests that the supplied glucose concentration might be enough during the night time under mixotrophic growth in addition to atmospheric CO₂ as well as light. However, the lower treatment efficiency with regard to hydroponic wastewater supplied with initial glucose concentration of 5 g/L might be due to the effect of non-sterilization, meaning that when glucose was supplied with relatively high concentration, the organic load was increased in the cultivation media and subsequently other microorganisms competed with microalgae to assimilate the source, and therefore affecting the treatment efficiency of TN and TP. On the contrary, the best removal efficiency of TN and TP were obtained for the initial glucose concentration of 5 g/L regarding aquaponic wastewater sample compared with that of 2.5 g/L. A probable explanation to this could be due to the low organic compounds of aquaponic wastewater sample compared to that for hydroponic wastewater sample. Thus, the addition of 5 g/L glucose to the medium increased the organic load in the media and the growth of microalgae, and simultaneously the treatment efficiency of TN and TP, unlike that for 2.5 g/L indicating that the glucose was not enough for enhancing the growth of microalgae during night time. Overall, it seems possible that the effect of non-sterilization resulted in lower treatment efficiency of TN and TP for the initial glucose concentrations of both 2.5 g/L and 5 g/L regarding both hydroponic and aquaponic wastewater samples in comparison with the results obtained for sterilized hydroponic and aquaponic
wastewater samples in experimental phase 1,2,3. Thus, more investigations regarding such conditions are needed in order to enhance the biomass production and simultaneously large cultivation facilities.
Chapter 5 TECHNO-ECONOMIC ASSESSMENT

5.1 General

This chapter presents details in connection to techno-economic assessment, focusing on three themes: background and motivation, methodology, results and discussion.
5.2 Introduction

The growing interest towards decarbonizing the world energy systems has encouraged research on exploring a new paradigm in the energy sector, particularly biofuels resources, that would accelerate the prospect of achieving the sustainable development goals (SDGs). Recently, biofuels have emerged as a powerful source that can be used to replace petroleum based-energy resources, which will come in the light of energy security and mitigation of greenhouse gases (GHGs) emission. Nevertheless, first-generation biofuels, commonly bioethanol and biodiesel, and second-generation biofuels, which is produced from non-food biomass, have received many critical opprobrium, particularly with regards to their impacts on food security and land use (Moore, 2008).

Considering their limitations, a considerable number of techno-economic assessments have demonstrated that third-generation biofuels through the utilization of microalgae can circumvent some of the cons associated with the first-and-second-generation biofuels. For instance, among the merits of microalgae is that they are capable of producing a huge amount of oil throughout the year and that they have a high rate in terms of the absorption and uptake of CO₂ (Brennan & Owende, 2010). In connection to the land use, microalgae do not require vast areas of land to be cultivated, meaning the issue of food security will not be compromised (Xin, Hong-ying, Ke, & Ying-xue, 2010). Microalgae can be grown in different types of wastewater without the need for high water usage compared with terrestrial crops, thus minimizing the burden on freshwater resources (Brennan & Owende, 2010). Furthermore, the growth potential of microalgae is tremendous: the cell doubling time is in the range of 1-10 days (Schenk et al., 2008), and many species of microalgae have high lipid content, more than 50 percent of dry weight (Hu et al., 2008).

Recent development in biofuel production through the utilization of microalgae have highlighted the need for exploring the economic implications in connection to large industrial operation of biorefineries as it can play an important role for transforming the economics of industrial production. Biorefineries produce a variety of utilitarian products which can be integrated into industrial biotechnology and therefore accelerating such transformation. However, this integration cannot be accomplished without effective and concurrent conversion of a broad range of microalgal biomass feedstocks into affordable biofuels and other important byproducts. The production of high value products from microalgae biomass become an economic driver that
provides higher margins of incomes for supporting the production of low-value products, and therefore offering a positive energy balance for a profitable operation of biorefinery (OECD, 2011).

Currently a tremendous number of applications regarding microalgae have been explored in order to support the profitability of large-scale operation of biorefinery. For instance, microalgae serve as good nutritional source for human because they are rich sources of carbohydrates, protein, enzymes, fiber, and many minerals (vitamins). Another important benefit of microalgae is that many of their components are commonly used in cosmetics for different purposes, for example as thickening agents, water-binding agents, and antioxidants. Arthrospira and Chlorella are the common types of microalgae that are used in skin care market (Priyadarshani & Rath, 2012). Microalgae also can be used to both treat wastewater and produce biofuels (Priyadarshani & Rath, 2012). They are useful for a variety of animals such as fish (aquaculture), pets, and farm animals as they can be incorporated into the feed of these animals (Spolaore et al., 2006). Furthermore, microalgal biomass can be converted to produce renewable sources of energy, such as biodiesel, biogas, and biohydrogen through different conversion methods, which can be classified into biochemical conversion, chemical reaction, direct combustion, and thermochemical conversion.

Recently, there has been a renewed interest in exploring the commercial viability of cultivating microalgae under different cultural metabolisms (mixotrophic, heterotrophic, and autotrophic modes). For instance, heterotrophic cultivation of microalgae has been investigated for only small markets of high value products by using different microalgae species, mainly Chlorella spp., Nitzchia spp., and Schizochytrium spp., Haematococcus spp., Cryptothecodinium spp., (Chen & Chen, 2006). On the other hand, few microalgal species, mainly Chlorella spp. and Haematococcus pluvialis strains have been investigated commercially under mixotrophic mode of cultivation (Hudek et al. 2014). However, the production of bioenergy from heterotrophic and mixotrophic cultivation of microalgae cannot be successfully commercialized without an integrated biorefinery. A multiplicity of biofuels and other valuable products can be obtained by cultivating microalgae mixotrophically and heterotrophically (Bassi et al. 2014). In particular, heterotrophic mode of cultivation offers various products from cellular storage compounds, such as lipids and starch, to a large amount of hydrocarbons and polysaccharides that can boost the

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prospect of commercialization. On the other hand, mixotrophic mode of cultivation is of benefit with regard to producing pigments, lipids, proteins and alkanes. Considering the average biomass productivities obtained from the third phase of experimentation, it is possible to expect the composition of microalgae biomass and the likelihood of obtaining various products under mixotrophic, heterotrophic and autotrophic modes of cultivation. Therefore, this part of the research work intends to explore the economic implications of microalgal biofuels, focusing on the effect of different cultivation modes (mixotrophic, heterotrophic, and autotrophic modes) in accordance with the results obtained from the first part of the research.

5.3 State of the Arts

Studies assessing the environmental benefits and techno-economic feasibility of wastewater and waste CO\(_2\) for sustainable biofuels production based on microalgae have mostly focused on specific case studies. In addition, a limited number of large microalgae cultivation for the production of biofuels has been established regarding wastewater and waste CO\(_2\) for biofuel production owing to the techno-economic challenges and the sustainability of the available technologies. Thus, considerable efforts are needed to circumvent these challenges in order to ensure full operation of large-scale microalgae cultivation. The potential of environmental benefits of the utilization of wastewater for microalgae cultivation has been investigated in a few studies by using life cycle assessment technique.

For instance, Mu et al. (2014) stated that algal biofuels generated from the liquid removed from thickened sludge could have a small impact compared to petroleum-derived diesel in terms of the environment while algal biofuels produced from wastewater could reduce the environmental impacts associated with the wastewater treatment plants. Furthermore, Clarens et al. (2010) assessed three different municipal wastewater effluents as nutrients sources, namely nitrogen and phosphorus, and they have confirmed that wastewater could provide a tremendous source to microalgae cultivation. A pivotal study comparing the utilization of biomethane as vehicle fuel generated from microalgae cultivated in wastewater with the use of compressed natural gas and conventional wastewater treatment by using pilot-scale data, Maga (2017) reported that microalgae cultivated in this medium offers benefits to the environment in terms of climate, protection of fossil resources, and ozone depletion. On the other hand, particulate matter formation, photochemical
oxidant formation, water deprivation, and eutrophication were found to be of significant impact with regards to the environment.

The studies presented thus far provide evidence that many benefits with regards to the environment in connection to microalgae cultivation onto wastewater can be highly attainable for downstream production of a renewable source of energy. However, there is a need for conducting a comprehensive environmental and economic assessment regarding the utilization of wastewater for microalgae cultivation in order to ensure a full characterization and quantification of the economic and environmental welfares of the system. This recommendation was stressed by Clarens et al. (2010) who argued that the availability of sustainable nutrients sources (Nitrogen, Phosphorus, and Carbon) for microalgal growth could have a tremendous effect regarding the life cycle metrics of microalgae cultivation which have been primarily underreported and underexplored thus far in the recent studies.

In connection to the economic assessment of the utilization of microalgae for production of biofuels, there are a considerable number of studies which have been published on the subject with various results that can be attributed to issues, such as process boundaries, type of cultivation system, variations in downstream conversion processing pathways, and core modeling assumptions (Campbell, Beer, & Batten, 2011; Clarens et al., 2010; Luo et al., 2010; Sander & Murthy, 2010; Sialve, Bernet, & Bernard, 2009). Another reason for such divergence can be related to various range of oil yields regarding microalgal biomass which directly affect the cost of microalgal biodiesel and other valuable products (Quinn & Davis, 2015; Sharma et al., 2015). These inconsistencies resulted in a wide divergence of results regarding the cost of producing microalgal biomass and biodiesel because of the early stage of development with regard to this technology (Schenk et al., 2008). Several lines of evidence suggest that the production of microalgae-based biofuels could be achieved with the development of large industrial operation of biorefineries that can transform the economics of industrial production. In a comprehensive literature survey conducted by Tapie and Bernard (1988) to assess the economic implications of large industrial operation of microalgae facility, they reported a biomass cost ranging from US$0.15 to US$4.00 per kg. Huntley & Redalje (2007) reported a cost of US$84 per barrel (2004 dollars) for oil production without considering any improvement in the technology. In the analysis
of the economics of three different microalgal systems (open ponds, horizontal tubular PBRs and flat panel PBRs) for a commercial 100 ha facility, Norsker et al. (2011) reported a biomass cost of 4.95, 4.15 and 5.96 € per kg for open ponds, horizontal tubular PBRs, and flat panel PBRs, respectively. Norsker also stated that irradiation conditions, mixing, photosynthetic efficiency of systems, cultural medium, and carbon dioxide costs are the most critical parameters with regard to biomass production cost. In an investigation of the economic implications of 100-hectare microalgal production facility for production of biocrude, ethanol, and animal feed, Beal et al. (2015) found that the best scenario case at market price of US$2 per liter of biocrude in line with benefits with regard to the environment and reducing burden on freshwater resources. To examine the possible success of both open pond and PBR cultivation systems according to Davis’ model, Richardson et al. (2012) described high probabilities of enterprise success of different scenarios where operating expenditures and capital expenses were decreased around 10% of their baseline values. In two of the most current and overarching economic assessment studies conducted by Jones et al. (2014) and Davis et al. (2016) to assess the downstream production costs of microalgal biofuel through microalgal fractionation and hydrothermal liquefaction, respectively, a biomass cost of US$474/ton and US$491/ton was reported, respectively.

Considering the effect of different cultivation modes, mixotrophic and heterotrophic culturing of microalgae have gained considerable attention recently in order to enhance the biomass production and subsequently supporting the profitability of large-scale operation of biorefinery. However, there is a relatively small body of literature that has investigated the commercial viability of cultivating microalgae under different cultural metabolisms, mainly mixotrophic and heterotrophic modes. Tabernero et al. (2012) conducted an economic assessment with regard to the microalgal biodiesel under heterotrophic culture, they reported a conservative production cost of US$1.4 kg⁻¹ and an optimized production cost of US$1.19 kg⁻¹ regarding microalgal biomass. For an optimized production cost an investment cost of US$0.68 kg⁻¹ year⁻¹ was added to the annual total production cost divided by production capacity while an investment cost of US$0.93 kg⁻¹ year⁻¹ was added to the conservative cost. Wijffels et al. (2010) estimated microalgal biomass cost of autotrophic cultivation mode in flat panel reactors using the conservative approach, and they stated a biomass cost of US$11.3 kg⁻¹ per hectare. On the other hand, an optimized cost of US$0.5 kg⁻¹ was reported taking into consideration some technical
issues, such as free supplementation of nutrients and CO\textsubscript{2} from waste biomasses, a 10% reduction of energy inputs, a 5 to 7% increase in photosynthetic efficiency of the microalgae strain, and the placement of photo-bioreactors in a location characterized by high levels of sunshine. Nevertheless, evaluations of the economic implications of mixotrophic biomass were not found in the literature. Therefore, there is a vital need for exploring the economic implications of different cultural metabolisms, particularly mixotrophic and heterotrophic modes of cultivation, for supporting the profitability of large-scale operation of biorefinery.

To better understand the potential benefits of microalgae cultivation for biofuels production, there is a need for assessing the commercial feasibility of mixotrophic and heterotrophic biomass. In this regard, according to the author’s stance, very few studies have been conducted to assess the economic implications of producing biomass under heterotrophic culture while no studies have found regarding mixotrophic mode of cultivation. In particular, the following research question need to be answered: What are the effects of different cultural metabolisms, particularly mixotrophic and heterotrophic cultivations, onto the economic assessment of microalgae-based biofuels problem set based on the results obtained from the first part of this research?

5.4 Objective

The objective of this part of research work was to conduct a techno-economic analysis with regard to the effect of different cultural modes of cultivation based on the results obtained from the first part of this research work, specifically in connection to the second phase of the experimentation.
5.5 Methods

The current section gives a full description of the methods that have been used and followed to reach the objective in second part of the current research work.

5.5.1 Cultivation

Based on the results of the experimental runs, experimental phase two has been upscaled in order to assess its techno-economic feasibility. In this phase of the experimentation, which was conducted in order to assess the effect of different cultural metabolisms (autotrophic, heterotrophic, and mixotrophic conditions) onto the growth of microalgae, removal of nutrient, and production of biomass, the observation was that *C. vulgaris* can grow autotrophically, heterotrophically, and mixotrophically. Specifically, the highest removal efficiency, biomass production, and growth rate was obtained for mixotrophic mode of cultivation followed by heterotrophic condition, then autotrophic condition in hydroponic wastewater. According to these outcomes, the phase was upscaled for the sake of assessing its economic implications based on the results obtained from hydroponic wastewater used as a cultivation medium.

The techno-economic model assumed the cultivation of *C. vulgaris* in a plastic bag PBRs, and it is based on a model characterized by Zhu et al. (2018). Biomass productivities obtained from the experimental phase number three were upscaled by a factor of 5 to reach a biomass productivity of 24.63 g/m²/d, 23.76 g/m²/d, 3.55 g/m²/d on an ash-free dry weight (AFDW) for mixotrophic, heterotrophic, and autotrophic conditions, respectively. These biomass productivities would achieve a constant biomass yield of 99.7 tones per day for mixotrophic condition, 96.2 tones per day for heterotrophic condition, and 14.4 tones per day regarding autotrophic condition. According to Zhu et al. (2018), the areal productivity in conjunction with plastic bag PBR systems diverges based on a variety of conditions, such as bag dimensions and temperature. For instance, a biomass productivity ranging from 5 to 35 g/m²/d has been reported by Ting et al. (2017) and Zittelli et al. (2013). Based on the aforementioned information with regard to biomass productivity, the scale-up factor of 5 was chosen in order to get an approximate values of biomass productivities that would be suitable for large microalgae cultivation facility and to be in line with the ranges found in the literature. Harvesting density of biomass was assumed to be 2 g/L. In accordance with industrial input and commercial feasibility, the cultivation area was assumed to be 1000 acres. The
PBRs energy requirements were assumed to be mainly for water circulation (287 W/m³), aeration (340 kWh/short tonne AFDW), and algae dewatering (Zhu et al., 2018).

5.5.2 Techno-economic Assumptions

Techno-economic model for this research work was conducted in the light of the costs presented by Zhu et al. (2018) Wijffels et al. (2010), and Xu et al. (2006). These costs include direct and indirect capital investment cost with regard to microalgal facility, fixed operating costs, and variable operating costs, biomass production cost, and the market values of the products. According to Zhu et al. (2018), total capital investment (TCI) per annual AFDW short ton algae is 1137 US$ which is equivalent to 1253.328 US$ per annual AFDW metric ton. This value was used to calculate the total biomass production cost. Following to that, microalgal biomass was assumed to be refined into various products for bulk chemical markets. Sajadian, Morowvat, & Ghasemi (2018) assumed different biomass compositions in accordance with the growth mode of microalgae (mixotrophic, heterotrophic, and autotrophic conditions). For mixotrophic growth mode, a composition of 44.25% lipids, 31.2% protein, 19.44% carbohydrates, and other products of 5.11% were assumed while a composition of 48.68% lipids, 33.55% protein, 13.16% carbohydrates, and other products of 4.61% were assumed. In connection to autotrophic mode, a composition of 34.01% lipids, 41.7% protein, 17.41% carbohydrates, and other products of 6.88% were assumed. Wijffels et al. (2010) and Xu et al. (2006) also assumed different compositions of microalgal biomass for autotrophic and heterotrophic mode of cultivation in line with their market prices. The two analyses assumed that 25% of the lipids fraction can be used for producing bulk chemicals with a market value of 2.5 US$/kg while the remaining can be utilized for producing a renewable source of energy (biodiesel) with a market value of 0.72 US$/kg. With regard to the protein fraction, it was assumed that 20% of a water-soluble fraction with a market value of 6.3 US$/kg and 80% of a water insoluble fraction with a feed value of 0.95 US$/kg. A 100% of carbohydrates fraction was assumed to have a market value of 1.26 US$/kg. These costs were assumed for this research work to calculate the total market values of the products and compare it with the total production cost in order to estimate the net profit. A microalgal nitrogen and phosphorous compositions were assumed to be 9.3 wt% AFDW and 0.6 wt% AFDW, respectively (Davis et al., 2016). Phosphorous removal cost of 42 US$/lb (Bashar, Gungor, Karthikeyan, & Barak, 2018) and a nitrogen removal cost of 2.52 US$/kg (Perez-Garcia & Bashan, 2015) were
used to calculate the cost saving of both phosphorous and nitrogen, respectively. These costs are the cost of nitrogen and phosphorous removals from wastewater using biological treatment processes. Furthermore, techno-economic model takes into account all economic indicators using LCA.

5.6 Results and Discussion

This section presents details regarding biomass productivities and scalability, biomass product costs, market values of the products, net profits and their discussion.

5.6.1 Biomass Productivities and Scale-up

Biomass productivities for mixotrophic, heterotrophic, and autotrophic modes of cultivation obtained from the second phase of the experimentation were upscaled by a factor of 5 and used to calculate the total biomass yield per year (Table 5.1). From data presented in Table 5.1, it is apparent that the highest biomass production was obtained for mixotrophic culture: 32900 tone/year, followed by a biomass yield of 31700 tone/year for heterotrophic growth while the lowest biomass yield was obtained for autotrophic growth. Under mixotrophic culturing of microalgae, there are two distinctive processes, photosynthesis and aerobic respiration which means that microalgae use light as energy source while they use both organic and inorganic carbon as carbon source. This might be the major reason for the highest biomass production obtained for mixotrophic growth. On the other hand, microalgae use organic carbon as the only source for both energy and carbon to support the growth of heterotrophic microalgae which could be the reason for enhancing the biomass production. However, during autotrophic culturing of microalgae, inorganic carbon and light were used as both carbon source and energy for supporting the growth of microalgae and enhancing the biomass production. The lower biomass yield obtained during autotrophic growth compared with heterotrophic and mixotrophic growth might be due to photoinhibition that indicates light deficiency as some microalgal cell do not receive enough light because of shading phenomena. More explanation is provided in the first part of this research work (experimental phase 2).
Table 5.1: Biomass Productivities obtained from the experiment with scalability

<table>
<thead>
<tr>
<th>Growth Mode</th>
<th>Biomass Productivity based on experiment (g/l/d)</th>
<th>Biomass Productivity after Upscaling (g/m²^2/d)</th>
<th>Biomass Yield (tone/y AFDW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixotrophic Mode</td>
<td>0.1108</td>
<td>24.63</td>
<td>3.29E+04</td>
</tr>
<tr>
<td>Heterotrophic Mode</td>
<td>0.107</td>
<td>23.8</td>
<td>3.17E+04</td>
</tr>
<tr>
<td>Autotrophic Mode</td>
<td>0.016</td>
<td>3.6</td>
<td>4.74E+03</td>
</tr>
</tbody>
</table>

5.6.2 Biomass Production Cost, Market Values of the Products, and Net Profit

Biomass compositions, percentages of the products from biomass compositions and their markets prices were used to calculate the total biomass production cost, total value of the products, and the net profit. Results regarding biomass production and products values for mixotrophic, heterotrophic, and autotrophic modes are shown in Table 5.2, Table 5.3, and Table 5.4, respectively. With regard to mixotrophic mode, annual total production cost and products values are 45.4 MMUSS/y and 71.8 MMUSS/y, respectively, resulting in a net profit of 26.4 MMUSS/y as presented in Table 5.2. What stands out in Fig. 5.1 are that biofuels and nutritious supplements from soluble protein accounted for the highest biomass production cost and market values of the products, respectively. Nitrogen removal resulted in a cost saving of 7.71 MMUSS/y while 18.4 MMUSS/y of saving accounted for phosphorous removal. The total yearly production cost and product values in conjunction with heterotrophic culture were 43.8 MMUSS/y and 69.92 MMUSS/y while the net profit was 26.1 MMUSS/y (Table 5.3). From Fig. 5.2, it can be seen that by far the greatest contribution with regard to biomass production cost was accounted for biofuels from lipids followed by feeds from insoluble protein with a small share from nutritious supplements from soluble protein. On the other hand, nutritious supplements from soluble protein accounted for the highest market values of the products with a small contribution from bulk chemicals from carbohydrates. A saving of 7.44 MMUSS/y was for nitrogen removal while 17.7 MMUSS/y was the saving for phosphorous removal (Table 5.3). The results with regard to autotrophic mode, as shown in Table 5.4, indicate that a 6.55 MMUSS/y was the total annual production cost while a 10.68 MMUSS/y was the yearly total market value of the products. This resulted in a net profit of 4.12 MMUSS/y annually. Nitrogen and phosphorous removal resulted in a cost saving of 1.11 MMUSS/y and 2.65 MMUSS/y, respectively. From Fig. 5.3, we can see that
the production cost is dominated by feed from insoluble protein and biofuels while products values are dominated by nutritious supplement from soluble protein as well as feed from insoluble protein. Comparing between mixotrophic, heterotrophic, and autotrophic modes are presented in Fig. 5.4. What can be seen from this figure is the highest product values with regard to both mixotrophic and heterotrophic modes compared to the total biomass production cost. These two figures resulted in a quite higher positive net profit. On the contrary, a small net profit was obtained for photo-autotrophic growth.

<table>
<thead>
<tr>
<th>Products</th>
<th>Percentage of biomass composition (%)</th>
<th>Biomass yield (MM kg/y on an AFDW)</th>
<th>Biomass production cost (MMUS$/y)</th>
<th>Price (US$/kg)</th>
<th>Product value (MMUS$/y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodiesel</td>
<td>33.1875</td>
<td>10.92</td>
<td>15.08</td>
<td>0.72</td>
<td>7.86</td>
</tr>
<tr>
<td>Bulk chemicals from lipids</td>
<td>11.0625</td>
<td>3.64</td>
<td>5.027</td>
<td>2.5</td>
<td>9.1</td>
</tr>
<tr>
<td>Nutritious supplements from soluble protein</td>
<td>6.24</td>
<td>2.05</td>
<td>2.84</td>
<td>6.3</td>
<td>12.93</td>
</tr>
<tr>
<td>Feeds from insoluble protein</td>
<td>24.96</td>
<td>8.21</td>
<td>11.3</td>
<td>0.95</td>
<td>7.8</td>
</tr>
<tr>
<td>Bulk chemicals from carbohydrates</td>
<td>19.44</td>
<td>6.39</td>
<td>8.8</td>
<td>1.26</td>
<td>8.06</td>
</tr>
<tr>
<td>Others</td>
<td>5.11</td>
<td>1.68</td>
<td>2.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TN removal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.71</td>
</tr>
<tr>
<td>TP removal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.4</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>31.21</td>
<td>45.4</td>
<td>71.8</td>
<td></td>
</tr>
<tr>
<td>Net Profit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.4</td>
</tr>
</tbody>
</table>
Figure 5.1: Biomass production cost and market value of each product broken out by major contributions regarding mixotrophic growth

Table 5.3: Values of biomass production cost and the market prices of the products obtained from heterotrophic microalgae

<table>
<thead>
<tr>
<th>Products</th>
<th>Percentage of biomass composition (%)</th>
<th>Biomass yield (MM kg/y on an AFDW)</th>
<th>Biomass production cost (MMUS$/y)</th>
<th>Price (US$/kg)</th>
<th>Product value (MMUS$/y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodiesel</td>
<td>36.51</td>
<td>11.59</td>
<td>16</td>
<td>0.72</td>
<td>8.34</td>
</tr>
<tr>
<td>Bulk chemicals from lipids</td>
<td>12.17</td>
<td>3.86</td>
<td>5.34</td>
<td>2.5</td>
<td>9.66</td>
</tr>
<tr>
<td>Nutritious supplements from soluble protein</td>
<td>6.71</td>
<td>2.13</td>
<td>2.94</td>
<td>6.3</td>
<td>13.41</td>
</tr>
<tr>
<td>Feeds from insoluble protein</td>
<td>26.84</td>
<td>8.51</td>
<td>11.77</td>
<td>0.95</td>
<td>8.09</td>
</tr>
<tr>
<td>Bulk chemicals from carbohydrates</td>
<td>13.16</td>
<td>4.18</td>
<td>5.77</td>
<td>1.26</td>
<td>5.26</td>
</tr>
<tr>
<td>Others</td>
<td>4.61</td>
<td>1.46</td>
<td>2.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN removal</td>
<td></td>
<td></td>
<td></td>
<td>7.44</td>
<td></td>
</tr>
<tr>
<td>TP removal</td>
<td></td>
<td></td>
<td></td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>30.27</td>
<td>43.8</td>
<td>69.92</td>
<td></td>
</tr>
<tr>
<td>Net Profit</td>
<td></td>
<td></td>
<td></td>
<td>26.1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.2: Biomass production cost and market value of each product broken out by major contributions regarding heterotrophic growth.

Table 5.4: Values of biomass production cost and the market prices of the products obtained from photo-autotrophic microalgae

<table>
<thead>
<tr>
<th>Products</th>
<th>Percentage of biomass composition (%)</th>
<th>Biomass yield (MM kg/y on an AFDW)</th>
<th>Biomass production cost (MMUS$/y)</th>
<th>Price (US$/kg)</th>
<th>Product value (MMUS$/y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodiesel</td>
<td>25.5075</td>
<td>1.21</td>
<td>1.67</td>
<td>0.72</td>
<td>0.87</td>
</tr>
<tr>
<td>Bulk chemicals from lipids</td>
<td>8.5025</td>
<td>0.4</td>
<td>0.56</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Nutritious supplements from soluble protein</td>
<td>8.34</td>
<td>0.395</td>
<td>0.55</td>
<td>6.3</td>
<td>2.49</td>
</tr>
<tr>
<td>Feeds from insoluble protein</td>
<td>33.36</td>
<td>1.58</td>
<td>2.19</td>
<td>0.95</td>
<td>1.5</td>
</tr>
<tr>
<td>Bulk chemicals from carbohydrates</td>
<td>17.41</td>
<td>0.83</td>
<td>1.14</td>
<td>1.26</td>
<td>1.04</td>
</tr>
<tr>
<td>Other</td>
<td>6.88</td>
<td>0.33</td>
<td>0.45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TN removal</td>
<td></td>
<td></td>
<td></td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>TP removal</td>
<td></td>
<td></td>
<td></td>
<td>2.65</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>4.42</td>
<td>6.55</td>
<td>10.68</td>
<td></td>
</tr>
<tr>
<td>Net Profit</td>
<td></td>
<td></td>
<td></td>
<td>4.12</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.3: Biomass production cost and market value of each product broken out by major contributions regarding photo-autotrophic growth

Figure 5.4: Comparison between mixotrophic, heterotrophic, and autotrophic modes in terms of total production cost, total products values, and net profit
5.7 Discussion

In general, higher profits were attributed to both mixotrophic and heterotrophic culturing of microalgae, with a small profit of that for autotrophic mode. This indicates that mixotrophic and heterotrophic cultivation offer many opportunities for boosting the commercial feasibility of producing biofuels. Considering the higher composition of lipid in heterotrophic culture (48.68 wt% AFDW) and mixotrophic cultivation (44.25 wt% AFDW), the production of biodiesel is highly attractive. The highest biomass obtained from mixotrophic mode might be a possible reason for the higher profit obtained from this economic assessment. This is based on the fact that mixotrophic microalgae grow both heterotrophically and autotrophically, which play an important role for enhancing the growth rate and biomass production of mixotrophic microalgae. Heterotrophic cultivation also showed a higher possibility of increasing the profit if large cultivation facility is built since it offers a reasonable amount of lipids (48.68 wt% AFDW) which is a key factor for biodiesel production. Heterotrophic microalgae are completely dependent on carbon source as they use it as both carbon and energy source, which is a merit because it eliminates the cost of light supplementation that is needed for both autotrophic and mixotrophic microalgae. While autotrophic microalgae also offer many advantages with regard to the environment, the economic assessment showed that huge improvements in terms of enhancing the biomass production are needed to boost the prospects of commercialization. The lower biomass production obtained from autotrophic microalgae could be a probable reason for the lower profit obtained from this mode of cultivation. This lower biomass could be possibly enhanced with the enhancement of photosynthetic efficiency and avoidance of photoinhibition that significantly affect autotrophic microalgae.

Despite the highest cost of biomass production with regard to large scale microalgae facility, more opportunities exist if the current technology is developed and if more products are explored in order to reduce the cost of biomass production and refinery. The economic analysis also showed that additional benefits with regard to the environment can be attained if wastewater is used as a cultivation medium. Using wastewater as a cultivation medium for microalgal growth is significantly important as can play a simultaneous role for removing the nutrients load in wastewater and producing biomass for sustainable biofuel production as well as reducing the burden on freshwater resources. Based on the fact that removal of nitrogen and phosphorous from
wastewater through biological removal technologies is an expensive process, the utilization of microalgae for the purpose of removing such nutrients will tremendously eliminate such expensive processes. This can be achieved through the integration of microalgae production system with wastewater treatment leading to higher saving in cost for both phosphorous and nitrogen removals. A successful integration of microalgae cultivation facility with wastewater treatment plants will play a big role for reducing environmental externalities and simultaneously boosting the prospects of attaining SDG 6, 14, 13.

Techno-economic assessment (TEA) also showed that there is a paramount need for refining microalgal biomass into various products in order to reduce the cost of biomass production while simultaneously transforming the economics of biorefinery. For instance, with the optimization of biomass production cost and the enhancement of the lipids content regarding heterotrophic and mixotrophic microalgae, it is possible to transform the entire lipids into biodiesel and to subsequently reduce the cost of biomass production. Such enhancement would play a critical role for boosting the commercial feasibility of microalgal biodiesel and even makes it competitive with petroleum biodiesel. However, based on the results obtained from this economic assessment, it can be concluded that microalgal biofuels are not competitive with traditional fossil fuels even with large scale facility due to huge investment onset. For instance, the cost of biomass based on this analysis is 1.38 US$/kg. If we compare it with average market price of petroleum biodiesel, which is 0.8344 US$/kg, it is clear that the value of producing biodiesel from petroleum is lower than that of biofuels. This economic assessment is also in agreement with other studies found in the literature (Jones et al., 2014; Davis et al., 2016; Tabernero et al., 2012; Wijffels et al., 2010) indicating that microalgal heterotrophic and autotrophic biofuels could be possibly successful with the development of the current technologies and exploration of other valuable products. Considering the huge cost of biomass production that can be attributed to issues, such as harvesting cost and bioreactors set up, it is of great importance to comprehensively develop new technologies that would reduce the cost of production and enhance the profitability of large microalgae facility.

The effective design of photobioreactor in line with a comprehensive assessment of different mathematical models with regard to microalgae biomass production process indicating the content of useful compounds of microalgal biomass are very critical issues that can affect the
cost of bioreactor. Enhancing the content of utilitarian compounds is a vital strategy for reducing the cost of biomass production and successfully boosting the prospects of commercialization. For the effective design of photobioreactor, irradiation conditions, mixing, photosynthetic efficiency of the system, cultural medium selection, and carbon dioxide supplementation and cost are some of the critical issues regarding photo-autotrophic and mixotrophic biofuels as they significantly affect the biomass production cost. Strain selection and subsequent development of appropriate cultivation system, harvesting, and downstream conversion processes are also another important factor for a successful biofuel system (Davis et al. 2011).

Based on the results obtained from both first part and second part of this research work, mixotrophic mode of cultivation showed a higher biomass production, growth rate, and the highest net profit compared with other modes of cultivation. Considering the simultaneous assimilation of both organic and inorganic carbon as source of carbon and light as energy source, it is of paramount importance to investigate closely the commercial success of mixotrophic biofuels in a large scale biorefinery. A successful implementation of mixotrophic biofuels will ensure a full utilization of comparative advantage. This is even more attractive, particularly when microalgae production facility is integrated with waste biomasses (wastewater and waste CO\(_2\)) that would contribute toward saving the environment. A successful integration of microalgal biofuels production facility with waste biomasses will play a big role regarding CO\(_2\) mitigation while simultaneously boosting the prospect of attaining the sustainable development goals. This will also reduce the cost of large industrial operation of biorefineries as it can play an important role for transforming the economics of industrial production.
Chapter 6 CONCLUSION AND RECOMMENDATION

The present study was undertaken to evaluate the use of microalgae for bioremediating wastewater from greenhouse farm and for producing biomass and to explore the economic implications of microalgal biofuels, focusing on the effect of different cultivation modes. The current research work has provided a deeper insight into the potential of microalgae for bioremediating hydroponic and aquaponic wastewater and for producing biomass under different conditions. This could make a positive contribution to the existing wastewater treatment technologies and simultaneously contribute towards boosting the prospects of the use of wastewater in microalgae cultivation for sustainable production of biofuels.

Two different samples of greenhouse farm wastewater, hydroponic and aquaponic raw wastewater, were efficiently treated in terms of total nitrogen and total phosphorous by green microalgae C. vulgaris. The best results in terms of total nitrogen and total phosphorous removals over the test periods were observed for mixotrophic growth supplied with 2.5 g/l glucose and atmospheric CO₂ showing reasonable removals of TN (98.5%), TP (99.99%) for hydroponic
wastewater sample, and TN (98.5%), TP (99.9%) for aquaponic wastewater sample. The reason for the stimulated increase in *C. vulgaris* and nutrients removals efficiencies in mixotrophic conditions compared with heterotrophic and autotrophic conditions is due to the simultaneous assimilation of both organic (glucose) and inorganic (atmospheric CO$_2$) carbon sources as well as light by *C. vulgaris*. This extra organic source of carbon enhances the growth of microalgae strain and the removal capacity of total nitrogen and total phosphorous. The maximum biomass concentration and productivity were observed for mixotrophic conditions in both hydroponic and aquaponic wastewater showing a reasonable amount of biomass concentration (1.26 g/L) and biomass productivity (0.1108 g/L/d) for hydroponic wastewater, and biomass concentration and productivity of 0.99 g/L, 0.089 g/L/d for aquaponic wastewater. Moreover, the highest lipid content values were obtained under heterotrophic of that of 37 wt% AFDW in aquaponic wastewater sample and a 33% wt% AFDW in hydroponic wastewater sample. On the other hand, the highest lipid production was obtained under mixotrophic (0.374 g/L) growth followed by heterotrophic (0.341 g/L) growth in hydroponic wastewater sample. The reason for the highest lipid content and lipid production is due to the addition of glucose as an organic source of carbon that plays an essential role for enhancing both the biomass and lipid producing ability under both mixotrophic and heterotrophic growth. The results obtained from this research work may be of benefit to a comprehensive understanding of the utilization of microalgae for the sake of wastewater treatment and production of biomass under different cultivation modes. Based on the literature review, it can be concluded that the use of wastewater as a cultivation medium for microalgae growth, concurrent removal of nutrients and production of biomass under mixotrophic conditions is a plausible strategy for biological wastewater treatment technologies and for a sustainable production of bioenergy. In addition, both heterotrophic and mixotrophic are of benefit with regard to lipid producing ability of microalgae, a product which determines biofuels producing ability of microalgae.

The research work has also shown that nitrogen to phosphorous ratio (N:P) plays an important role with regard to the total removal efficiencies of nitrogen and phosphorous to the biomass production of microalgae. The best removal efficiencies throughout the duration of the test were noticed for N:P molar ratio of 8:1 displaying removals of TP (88%) and TN (85%) compared with that for N:P ratios of 16:1 and 24:1. Maximum values with respect to biomass
concentration was obtained for N:P molar ratio of 8:1 while biomass productivity was almost the same in all N:P molar ratios. These findings contribute in several ways to our understanding of the effect of N:P ratios and provide a basis for the effect of N:P molar ratios on microalgal biomass producing ability and nutrient removal from wastewater. This study also adds to the growing body of research that indicates nitrogen to phosphorous ratio is of paramount importance for maximizing the biomass production of microalgae and the total removal efficiency of nutrients from wastewater.

The study also has investigated the effect of non-sterilization on the treatment efficiency of total nitrogen and total phosphorous and on the biomass production by microalgae in connection to both hydroponic and aquaponic wastewater samples supplied with two different initial glucose concentration under mixotrophic growth. The best results in terms of total nitrogen and total phosphorous removals over the test periods were observed for the initial glucose concentration of 2.5 g/L in hydroponic wastewater sample and for the initial glucose concentration of 5 g/L in aquaponic wastewater sample showing reasonable removals of TN (73.33%), TP (86.56%), and TN (72%), TP (77.78%), respectively. The maximum biomass concentration and biomass productivity were observed for the initial glucose concentration of 5 g/L, 0.35 g/L and 0.02 g/L/d, respectively, compared to 2.5 g/L glucose in aquaponic wastewater sample. Moreover, maximum biomass concentration of 0.54 g/L and biomass productivity of 0.056 g/L/d were observed for the initial glucose concentration of 2.5 g/L in hydroponic wastewater sample. The effect of non-sterilization resulted in lower treatment efficiency of TN and TP and in lower biomass production for the initial glucose concentration of 2.5 g/L and 5 g/L regarding both hydroponic and aquaponic wastewater samples in comparison with the results obtained for sterilized hydroponic and aquaponic wastewater samples assessed in experimental phase 1,2,3. Thus, more investigations regarding such conditions are needed in order to enhance the biomass production, the treatment efficiency of nutrients, and simultaneously the large cultivation facility of biorefineries.

The study also has assessed the economic implications of microalgal biofuels, focused on the effect of different cultivation modes based on the results obtained from the experimentation. Cultivation modes assessed include mixotroph, heterotroph, and autotroph. Results from the economic assessment determined net profit for each type of cultivation mode. The best results
regarding the net profit were obtained for both mixotrophic and heterotrophic cultivations of 26.4 MMUSS/y and 26.1 MMUSS/y, respectively, while the net profit for autotrophic cultivation was 4.12 MMUSS/y. Sensitivity analysis shows that biodiesel and nutritious supplements from soluble protein have the greatest impact on the process economics with respect to mixotrophic cultivation while biodiesel and feeds from insoluble protein have the largest effect on the process economics in connection to both heterotrophic and autotrophic cultivations. The higher biomass and lipid concentration observed for mixotrophic and heterotrophic cultivation enhanced the economic feasibility of microalgal biofuels and subsequently boosted the prospects of commercialization. TEA also has shown that additional benefits for the environment can be attained if wastewater is used as a cultivation medium in order to bioremediate the waste and to produce biomass. Utilizing wastewater as a cultivation medium is a major step towards reducing the burden on freshwater resources while simultaneously making an attractive addition to the existing biological treatment technologies. The produced biomass from such waste is of benefit for the sustainable production of biofuels. TEA also showed that it is of great importance to refine microalgal biomass into various products for reducing the process economics of the entire system and for transforming the economics of industrial operations.

Suggestions for Future works:

1. Since the study was conducted in a laboratory scale with controlled conditions, it is proposed that future work should focus on examining the effect of seasonal temperature variations, effect of light intensity, and the effect of different carbon sources on nutrient removal and on biomass production. Assessing the effect of these conditions is fundamental step towards a successful production of biofuels and management of wastewater.

2. Mixotrophic cultivation seems to offer a reasonable removal in terms of nutrients and biomass production which would be a fruitful area of future research. Considering the huge amount of biomass that is required for ensuring a sustainable production of biofuels, mixotrophic cultivation could be a practical solution with regard to this issue. Organic and
inorganic carbon supplementation is one of the major factors for enhancing the growth of mixotrophic microalgae, indicating that different sources of carbon should be considered for future research. The same research efforts are needed for both heterotrophic and autotrophic cultivations.

3- Although highest lipid production and lipid content were obtained under mixotrophic and heterotrophic growth, it is of paramount importance to further investigate mixotrophic, heterotrophic, and autotrophic cultivations under different conditions, particularly regarding the effect of different sources of carbon and the effect of light intensity in future research. Such examination would play an important role for accelerating the sustainable production of microalgal biofuels and simultaneously boosting the prospect of large cultivation facility of biorefineries.

4- Wastewater is one of the fundamental problems regarding the environment because it contains a lot of contaminants, particularly inorganic compounds such as nitrogen and phosphorus. The fact is that removing the inorganic nitrogen and phosphorus through the use of the existing wastewater treatment technologies is very difficult and costly. Therefore, the use of microalgae for the bioremediation of wastewater, for fixation of CO$_2$, and for production of biomass could be the most plausible solution regarding these issues. Based on that, further research is needed for exploring the possibility of integrating microalgal production facility with wastewater treatment. Such integration will play a vital role for reducing the environmental externalities and for boosting the prospects of achieving the sustainable development goals.

5- Although several life cycle analysis (LCA) and techno-economic assessment involving biofuels from microalgae have been conducted, mostly focusing on specific case studies, a limited number of large microalgal cultivation for the production of biofuels has been established regarding different types of wastewater and different sources of waste CO$_2$ for biofuel production owing to the techno-economic challenges and the sustainability of the available technologies. Thus, considerable efforts are needed to circumvent these challenges in order to ensure the full operation of large-scale microalgal cultivation. In
addition, CO₂ capture and storage using microalgae have gained considerable attention recently in order to minimize the effects of climate change and subsequently boost the prospect of achieving the SDGs. Nevertheless, there is a relatively small body of literature that has investigated the environmental benefits and the sustainability of carbon transportation regarding the use of waste CO₂ as a primary source of carbon in connection to the cultivation of microalgae. Accordingly, there is a need for a better understanding of the potential economic and environmental benefits/impacts of the utilization of microalgae for bioremediation of wastewater and mitigation of waste CO₂ for sustainable production of biofuels, and a need for structured approach in exploring and modeling the sustainability implications regarding the use of microalgae cultivation with different types of wastewater and their characterizations, and different sources of CO₂ as well as CO₂ delivery and its utilization efficiency with respect to the large-scale production of bioenergy. Addressing such important matters in future research would be of benefit to the industry as well as to the researchers.
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