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EFFECTS OF DIFFERENT COMBINATION OF RED, GREEN AND BLUE LED LIGHT SEPRCTRUMS ON THE GROWTH OF GREEN ALGAE CHLAMYDOMONAS REINHARDTII (CHLOROPHYTA)

MASTER OF SCIENCE

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Effects of different combination of red, green and blue LED light spectrums on the growth of green algae *Chlamydomonas reinhardtii* (Chlorophyta) submitted by **Dina Nabil Mohammad ALJAMILI** and defended before the Examining Committe Members listed below in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, Institute of Graduate Studies of Bolu Abant Izzet Baysal University in 2.05.2023** by

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ABSTRACT

EFFECTS OF DIFFERENT COMBINATION OF RED, GREEN AND BLUE LED LIGHT SPECTRUMS ON THE GROWTH OF GREEN ALGAE CHLAMYDOMONAS REINHARDTII (CHLOROPHYTA)
MSC THESIS
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The positive effects of LED light source recently used in algae biotechnology on growth and metabolite biosynthesis in microalgae are reported in scientific literature. In this study, the effect of white, monochromatic and combinations of red and blue lights in different ratios on the growth of green algae species *Chlamydomonas reinhardtii* (Chlorophyta), which is a model organism with high economic value in biotechnology field, were investigated. Additionally, the effect of green light gradual supplementation into red:blue light combination (1:2) percental increase of 0, 7.5, 15, 22.5, 30, and 37.5% in expense of red and blue lights was studied to evaluate the effect of green light supplementation on the growth and chlorophyll concentration in *C. reinhardtii*.

The results showed that both growth rate and chlorophyll concentration were statistically higher under dichromatic red and blue light combinations of the ratios 1:2 and 2:1 with no statistical difference between the combinations. the order of growth rate was Red: Blue (1:2) > Red: Blue (2:1) > Red: Blue (1:1) > White > Red > Blue. Green light supplementation has not shown statistical differences from control group (red1: blue2). However, none of the green-supplemented cultures showed lower growth rate than control group despite having lower red: blue levels that reached 37.5%. Also, chlorophyll concentrations showed significantly higher levels in almost all green light supplemented groups than control.

The results support the high adaptation mechanism by *C reinhardtii* under different light illumination and the additive effect of combining blue and red light through their differential enhancement of growth. Moreover, green light compensation mechanism is indicated in case of low blue and red light.

KEYWORDS: Green Light, *Chlamydomonas Reinhardtii*, LED Light, Chlorophyll.

ÖZET

KIRMIZI, MAVİ VE YEŞİL LED ISIK DALGA BOYLARININ FARKLI ORANLARDA UYGULANMASININ CHLAMYDOMONAS REINHARDTII (CHLOROPHYTA) YEŞİL ALG TÜRNÜN BÜYÜMESİNE OLAN ETKİLERİ
YÜKSEK LİSANS TEZİ
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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
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Son yıllarda alg biyoteknolojisinde kullanılan LED ışık kaynağının mikroalglerde büyüme ve metabolit biyosentezi üzerindeki olumlu etkileri bilimsel literatürde yer almaktadır. Bu çalışmada, biyoteknoloji alanında ekonomik değeri yüksek bir model organizma olan yeşil alg türü *Chlamydomonas reinhardtii* (Chlorophyta)'nın büyümesi üzerine beyaz, monokromatik ve farklı oranlarda kırmızı ve mavi ışık kombinasyonlarının etkisi incelenmiştir. Ek olarak, yeşil ışığın kademeli olarak kırmızı:mavi ışık kombinasyonuna (1:2) yüzde 0, 7,5, 15, 22,5, 30 ve 37,5'lik artışa etkisi, kırmızı ve mavi ışıkların etkisini değerlendirmek için incelenmiştir. C. reinhardtii'deki büyüme ve klorofil seviyelerine yeşil ışık takviyesi.
Sonuçlar, hem büyüme hızının hem de klorofil konsantrasyonunun, 1:2 ve 2:1 oranlarındaki dikromatik kırmızı ve mavi ışık kombinasyonları altında istatistiksel olarak daha yüksek olduğunu ve kombinasyonlar arasında istatistiksel bir fark olmadığını gösterdi. büyüme oranı sırası Kırmızı:Mavi(1:2) < Kırmızı:Mavi(2:1) < Kırmızı:Mavi(1:1) < Beyaz < Kırmızı < Mavi idi. Yeşil ışık takviyesi, kontrol grubundan (red1:blue2) istatistiksel olarak farklılık göstermedi. Bununla birlikte, yeşil takviyeli kültürlerin hiçbiri, %37,5'e ulaşan daha düşük kırmızı:mavi seviyelerine sahip olmasına rağmen, kontrol grubundan daha düşük büyüme oranı göstermedi. Ayrıca klorofil konsantrasyonları, yeşil ışıkla desteklenmiş grupların neredeyse tamamında kontrol grubuna göre önemli ölçüde daha yüksek seviyeler gösterdi.
Sonuçlar, farklı ışık aydınlatması altında *C. reinhardtii* tarafından sağlanan yüksek adaptasyon mekanizmasını ve mavi ve kırmızı ışığın büyümeyi farklı şekilde geliştirmeleri yoluyla birleştirmenin ek etkisini destekler. Düşük mavi ve kırmızı ışık durumunda yeşil ışık tarafından bir dengeleme mekanizması da önerilmiştir.

ANAHTAR KELİMELER: Green Light, *Chlamydomonas Reinhardtii*, LED Light, Chlorophyll.

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LIST OF ABBREVIATIONS AND SYMBOLS

C. reinhardtii **:** *Chlamydomonas reinhardtii*

CO_2 **:** Carbondioxide

ATP **:** Adinosine triphosphate

NADPH **:** Nicotinamide-adenine dinucleotide phosphate

NADH **:** Nicotinamide-adenine dinucleotide

PSI **:** Potosystems I

PSII **:** Photosystem II

PQ **:** Plastoquinone

LHCI **:** Lightharvesting complex I

LHCII **:** Lightharvesting complex II

ROS **:** Reactive oxygen species

CBC **:** Calvin-Benson cycle

CEF **:** Cyclic electron flow

LEF **:** Linear electron flow

PAR **:** Photosynthetic active radiation

GA3P **:** Glyceraldehyde 3-phosphate

3-PGA **:** 3-phosphoglyceric acid

RuBP **:** Ribulose 1,5-bisphosphate

IBS **:** Irritable bowel syndrome

LED **:** Light emitting diode

ANOVA **:** Analysis of variance

BG-11 **:** Blue-green (culture medium)-11

H_2O **:** Water

$NaNO_3$ **:** Sodium Nitrate

K_2HPO_4 **:** Dipotassium hydrogen phosphate

$MgSO_4$ **:** Magnesium sulfate

$CaCl_2$ **:** Calcium chloride

Na_2EDTA **:** Disodium Ethylene diamine tetraacetic acid

OD **:** Optical density

RPM **:** Round per minute

°C **:** Centigrade degree

SD **:** Standard deviation

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1. INTRODUCTION

Humans have continuously understood the usefulness of algae and plants as renewable sources of food, pharmaceuticals, and biofuels. Agricultural technologies have been modified and improved continuously throughout human history to satisfy the evolving needs of civilization. Today's environmental changes and the arising challenges necessitate additional innovation to overcome and compensate the limitations. Algae, aquatic photosynthetic organisms, are of great interest in the current studies because of their diversity and their potential utility in a wide range of applications.

1.1 Microalgae

Microalgae are unicellular aquatic micro-organisms that has been used by humans for more than 2000 years. They are primarily photosynthetic and grow in different environmental settings notably in marine and freshwater ecosystems in which they account for the largest part of the ocean's biogeochemical cycling (1). Microalgae are part of the highly diverse group; algae include a wide range of organisms ranging from large size macro-algae (such as seaweeds) to microscopic organisms (like Diatoms). Nevertheless, microalgae include both prokaryotic microorganisms (such as Cyanobacteria) and eukaryotic microalgae (e.g. green algae) (2).

1.1.1 Evolution

Algae is a major group of photosynthetic eukaryotic aquatic organisms, but might also be terrestrial, that have been proposed to evolve through multiple Endosymbiotic events from different origins about 470 million years ago that resulted in a great diversity in their phenotypes (3). Endosymbiosis was initially proposed in 1905 and it describe the engulfment of prokaryote (Cyanobacteria) by eukaryotic host (figure 1.1). Cyanobacteria survived within the cytoplasm of their host since they produced beneficial metabolites for growth, and eventually settled

permanently inside cells and further developed into chloroplasts, plastids, and most importantly light harvesting organelles (4). The first endosymbiotic event is called primary endosymbiosis and green algae are thought to have diverged (5). Furthermore, the oxygen produced by these phototrophs is thought to contribute to critical changes in atmosphere and eventually, the diversion of living organisms (6).

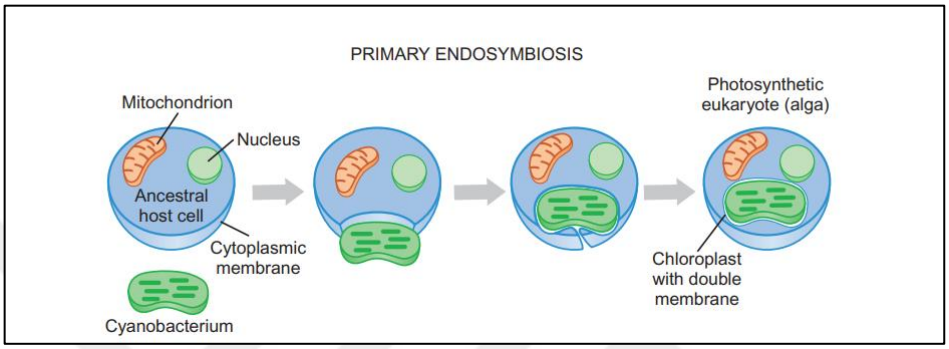


Figure 1.1. Simplified illustration of primary endosymbiosis (6).

While algae and plants are both photosynthetic autotrophic organisms, they are fundamentally distinct groupings. Algae lack many of the features seen in terrestrial plants, such as the roots, stems, and vascular tissues. Additionally, algae can be unicellular or multicellular, with vegetative or sexual reproduction (7). What distinguishes microalgae from plants is their rapid growth rate and the higher concentration of economically valuable compounds such as lipids or proteins; the quantity and type of compound generated vary according to growing conditions and algae species (8). The genetic and morphological diversity in algae resulted in their capability of adaptation and survival in different sets of environments. Figure 1.2 illustrates the phylogenetic tree of algae in evolutionary concept.

According to Abdullah et. al., (9), there are generally 6 groups of microalgae species which are: 1. Green algae (Chlorophyceae), 2. Red algae (Rhodophyceae), 3. Brown algae (Phaeophycae), 4. Yellow-green algae (Xnthophyceae), 5. Golden algae (Chrysophyceae), 6. Diatoms (Bacillariophyceae). The single-celled green algae (Chlorophyta) are regarded to be one of the major groups of algae known to science, and it include a diverse range of species found in different geographical

locations. This family of microalgae includes the most researched kinds of microalgae, such as *Chlamydomonas reinhardtii*, among others like *Chlorella* and *Haematococcus* species (10).

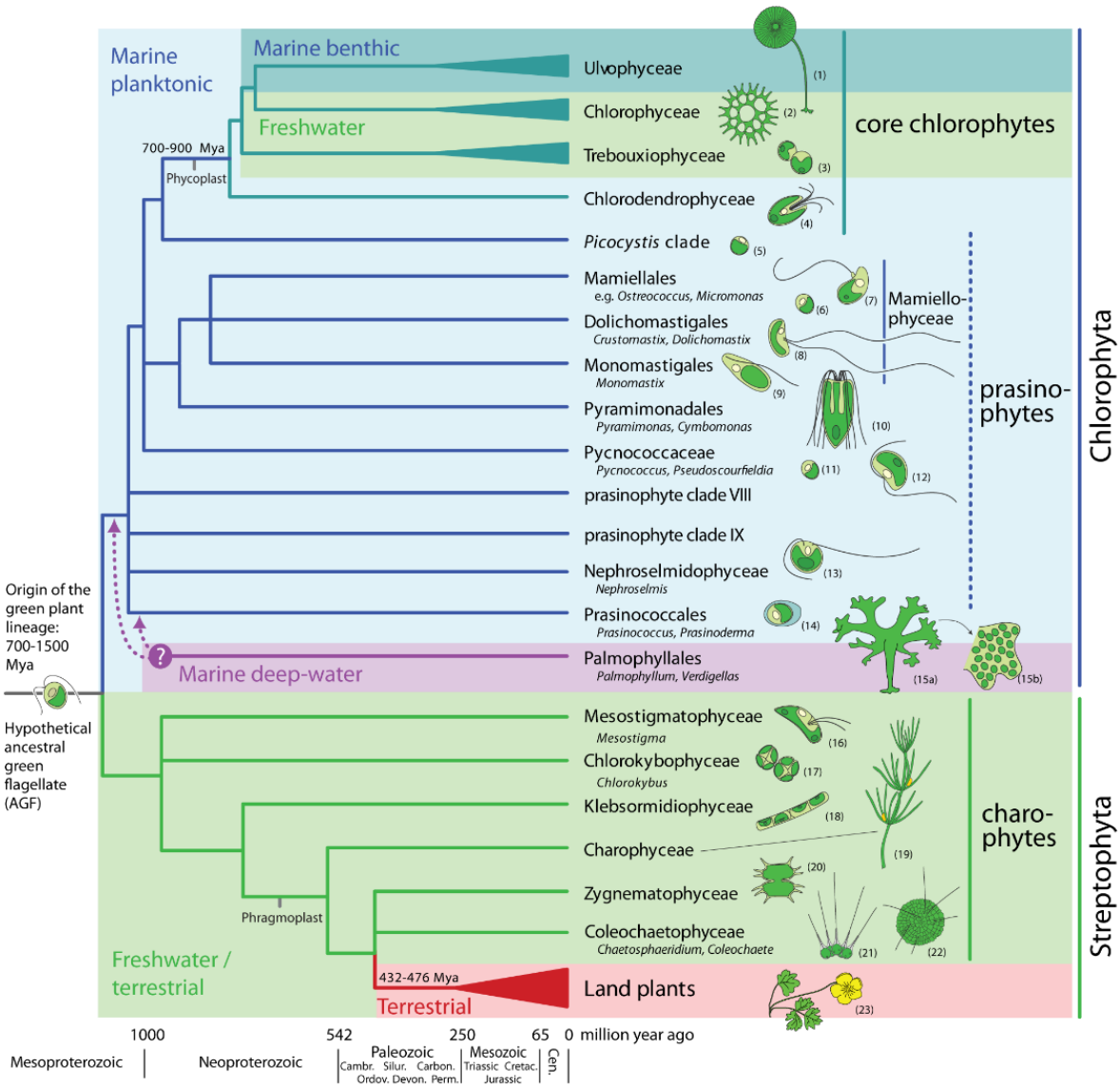


Figure 1.2. The phylogenetic tree of the green linage that is proposed to evolve from multiple endosymbiotic events. The placement of both different species of algae and land plants are shown in the figure (11).

1.1.2 Microalgae in Ecosystem

The aquatic ecosystem largely depends on microalgae as they contribute to about 50% of the global photosynthesis (12). The organic sinking carries carbon that has been fixed from the atmosphere into the ocean's interior, and therefor microalgae plays a major role in the atmospheric CO_2 levels and changes in the climate (1). As CO_2 dissolves into the water, it is captured by microalgae cells and converted into organic matter through the photosynthesis (figure 1.3) it is via this process that oxygen and carbohydrates are released, both of which are beneficial to the aquatic ecosystem (14). Also, microalgae play a critical role in sustaining the aquatic ecosystem production through the nitrogen fixation as well as enhancing the metabolites availability (15). The movement of nutrients between organisms and their habitats is referred to as nutrient cycle. Nutrients (most importantly nitrogen and phosphorous) are transformed from one chemical to another during this process. Microalgae are found to play a significant role in nitrogen cycle and are also influenced by nutrient levels in the environment (16).

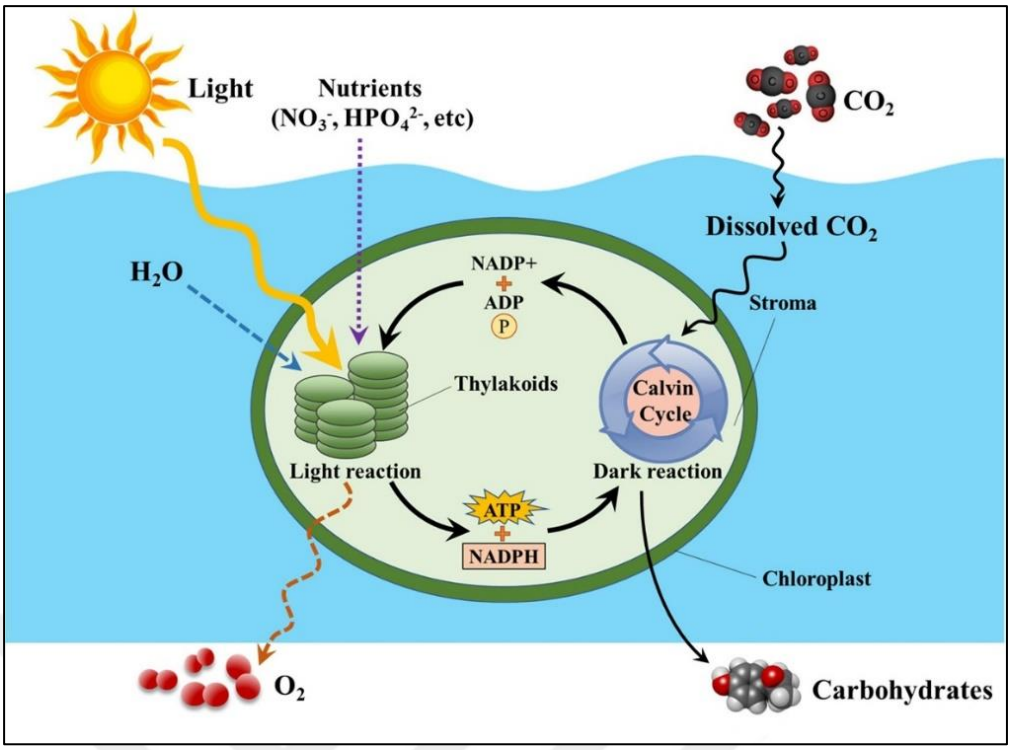


Figure 1.3. Microalgae photosynthesis in aquatic ecosystem. Microalgae absorbs the dissolved CO_2 from water in which it is evolved in Calvin cycle to produce carbohydrates, and also participates in nitrogen fixation (14).

1.1.3 Growth and Photosynthesis

The growth of microalgae (demonstrated in figure 1.4) consists of four phases (17):

1. Lag phase: no growth observed, maturing and adaptation to environment.

2. Exponential phase: fast multiplying growth, high nutrient consumption.

3. Stationary or plateau phase: steady growth and maximum cell density.

4. Death phase: cells' lysis, lack of nutrients.

Although almost all microalgae go through this life cycle, the length of each phase and other characteristics varies according to microalgae species and, most importantly, growth conditions.

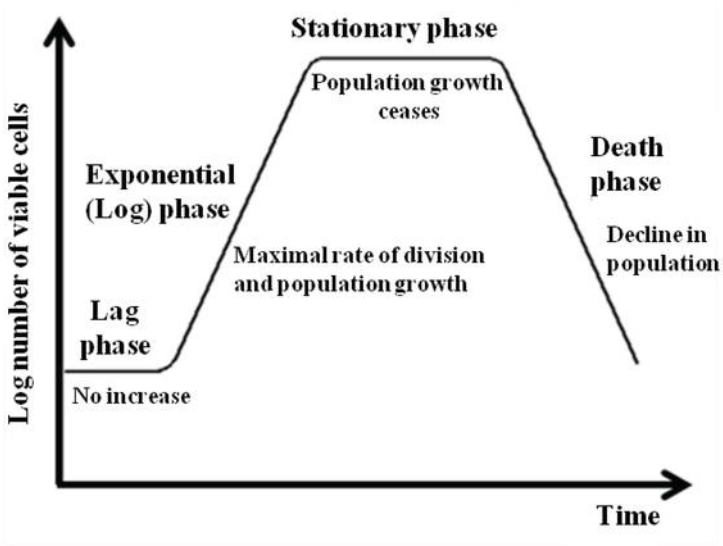


Figure 1.4. Microalgae growth curve and phases (18).

Photosynthesis is using the light in the conversion of water and carbon dioxide into sugars for microalgae growth and reproduction (19). In both autotrophic and mixotrophic growth, photosynthesis is critical and results in the production of oxygen, ATP, and NADPH. The growth phase (photosynthesis) is completely dependent on light as a source of energy, and the cells double their size many times to produce more daughter cells during their division (20). The photosynthesis can be broken down into two stages: 1) light-dependent reactions, and 2) light independent reaction (also called Calvin cycle or dark reactions).

A chloroplast takes up a significant amount of microalgae's cell volume and it is the organelle where photosynthesis occurs. Moreover, the pyrenoid, located inside the chloroplast, contains Rubisco and its main function is to concentrate inorganic carbon against the concentration gradient (21).

1.1.4 Light Dependent Reactions and Light Harvesting Pigments

The light is captured through light harvesting protein-pigment complexes found in the thylakoid membrane of microalgae called photosystem I and II (photosynthesis starts in PSII), the light energy is absorbed in the form of excited electrons that travels either in linear electron flow or cyclic electron flow and it results in reduction of plastoquinone (PQ) and the production of NADH and ATP (22).

PSI and PSII are parts of the light harvesting antennae and they are composed of protein complexes (known as light harvesting complex, LHCI and LHCII), pigments that account for absorbing light (like Chlorophylls), and other accessory pigments with different functions (like Carotenoids) (23). Furthermore, redox transporters like the Cytochrome b6/f complex are also part of the light harvesting system and functions in connecting the reaction centers, PSI and PSII, to one another and to also to the electron doners/ acceptors. ATP synthase enzyme is also part of the light harvesting system as it generates ATP at the end of light reaction of photosynthesis which is further used by the Calvin cycle (figure1.5) (24). An important point to mention is that the light harvesting antenna have a crucial role other than light capturing, which is the photoprotection against the harmful reactive oxygen species (ROS) and probably photoinhibition that occurs during photosynthesis when there is excess light energy, this function is mainly controlled by xanthophyll pigments (carotene yellow pigments) (25).

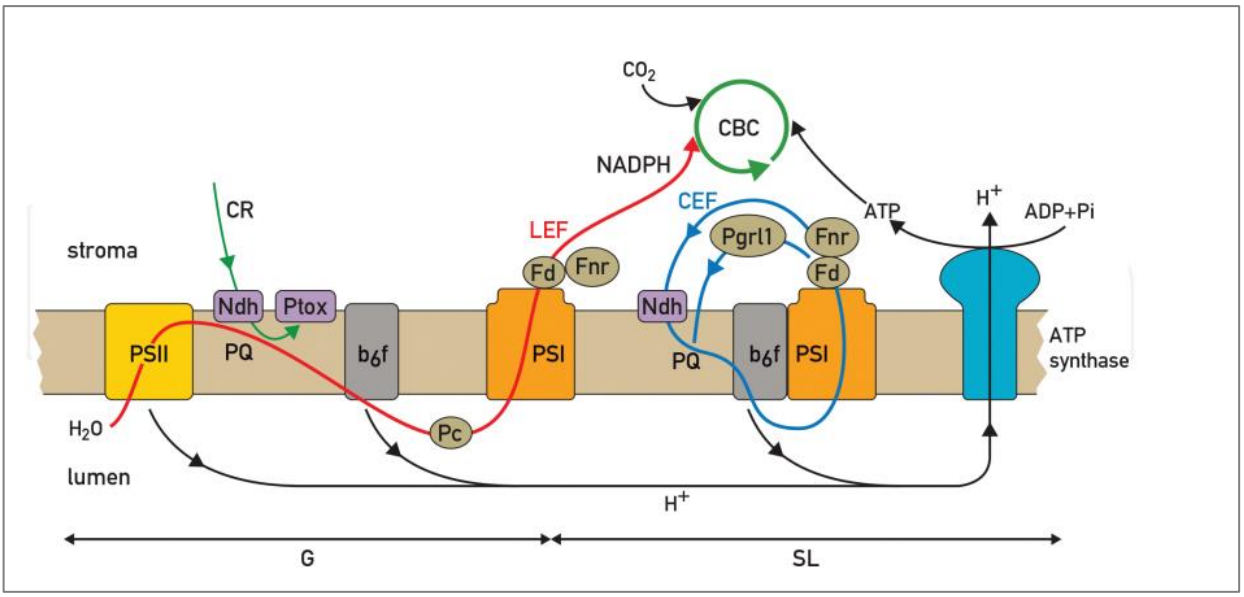


Figure 1.5. Light harvesting antenna in *C. reinhardtii.* Showing PSII, Cytochrome b6/f, PSI, ATP synthase along with the electron transport (26). CBC: Calvin-Benson cycle, CEF: cyclic electron flow, LEF: linear electron flow, PQ: plastoquinone.

In photosynthetic algae species, Chlorophyll, Carotenoids, and Phycobiliproteins are the three fundamental types of natural pigments that allow algae to be a possible source of natural colors. These natural pigments have many benefits including their strong antioxidant activity, anti-inflammatory, and neuroprotective properties in addition to their usage in other fields as pigments (27). Chlorophyll, green colored pigment, absorbs the red and blue wavelengths of the photosynthetic active radiation (PAR; between 400 to 700 nm wavelengths) of the light and converts them into chemical energy, whereas carotenoids serve as a bridge between the blue and red regions by absorbing the wavelengths of the green/blue regions (28).

For plants and algae, chlorophyll is one of the most valuable bioactive molecules since it is essential for light harvesting and photosynthesis process. Chlorophyll a and b are the two primary varieties and because of their structural variations, chlorophyll a is characterized by being a blue/green pigment and chlorophyll b is green/yellow pigment (29). The absorption spectrum of chlorophyll pigment is shown in figure 1.6.

When the light intensity is low during the linear growth phase and when cells are dense, a raise the chlorophyll content occurs to allow the algae to efficiently absorb the light available for its growth and biomass production to survive, this is due to the strong photo limitation conditions caused by low light intensity. However, When the cells of microalgae perform the photosynthesis more efficiently, less chlorophyll pigment is required (30).

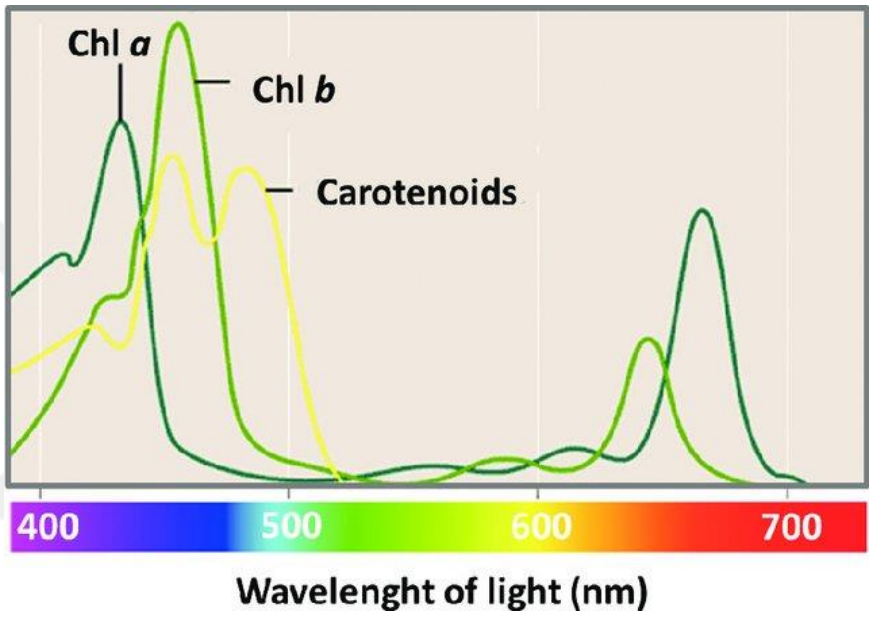


Figure 1.6. The absorption spectrum of chlorophyll a, chlorophyll b, and carotenoids (31).

Because photosystems are so effective at absorbing light, they must guard against excessive light absorption and photoinhibition. There are 3 lines for photo-protection against reactive oxygen species (ROS) produced under excess light (31):

1. Non photochemical quenching (NPQ): this is the first defense process, and it takes place in PSII and involves the action of different carotenoids pigments including xanthophylls (particularly zeaxanthin). The carotenoid pigments participate in a process called xanthophyll cycle (figure 1.7), correlated to chlorophyll a, in which it dissipates the excess light in the form of heat through chemical reactions.

2. Antioxidant compounds and enzymes the scavenge the ROS produced during photosynthesis.
3. Alternative electron flow in the photosystem (cyclic electron flow) can also reduce the excess production of ROS.

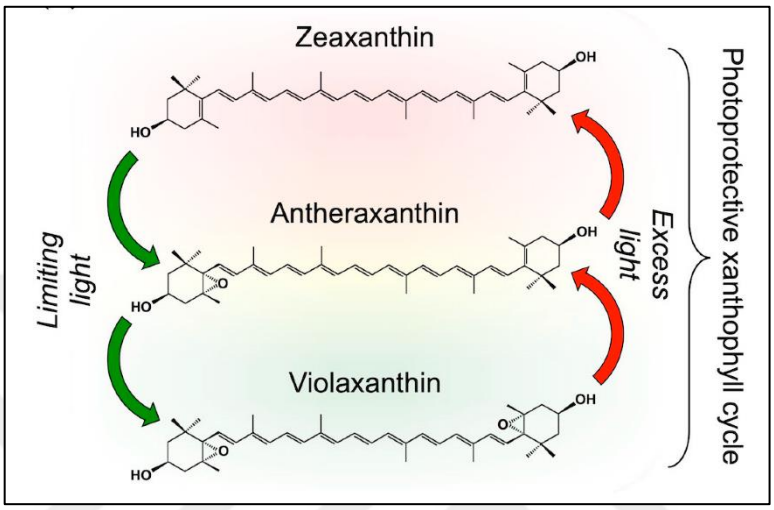


Figure 1.7. The photoprotective xanthophyll cycle. Xanthophylls not only function in dissipating the excess light in the form of heat, but also in harvesting light and transferring it to chlorophyll-a pigment for photosynthesis (32).

Furthermore, various spectrum photons are found to have different effects on certain sensory photoreceptors in algae that regulate the photosynthesis process, photo orientation and development. There are five major types of photoreceptors in algae and they are: BLUF-proteins, cryptochromes, phytochromes, phototropins, and rhodopsins. These photoreceptors sense variations in light intensity and wavelength and thus alter the algae movement behavior or change the physiological responses through signal transduction cascades (33).

Cryptochromes and phototropins photoreceptors respond to the blue wavelength resulting in multiple gene expression and metabolic processes (34). Genes of red-light absorbing photoreceptor phytochrome were not found in *C. reinhardtii*. However, at least one photoreceptor is found to be related to absorbing red light including blue light absorbing cryptochromes (35).

1.1.5 Carbon Assimilation

Calvin-Benson cycle is the second part in the photosynthesis, and it is not dependent upon light and thus also called “The dark reaction or cycle”. The vitality of this process is because it is only source of organic carbon for the generation of new biomass. This process utilizes the ATP and NADPH produced through the electron transfer by PSI and II in the reduction of CO_2 (36). The Calvin cycle consists of multiple enzymatic processes that use 9 molecules of ATP and 6 molecules of NADPH to make one molecule of GA3P that is the precursor of glucose sugar (37). This cycle can be divided into three stages (figure 1.8): 1) carbon fixation, 2) reduction stage, and 3) regeneration of ribulose.

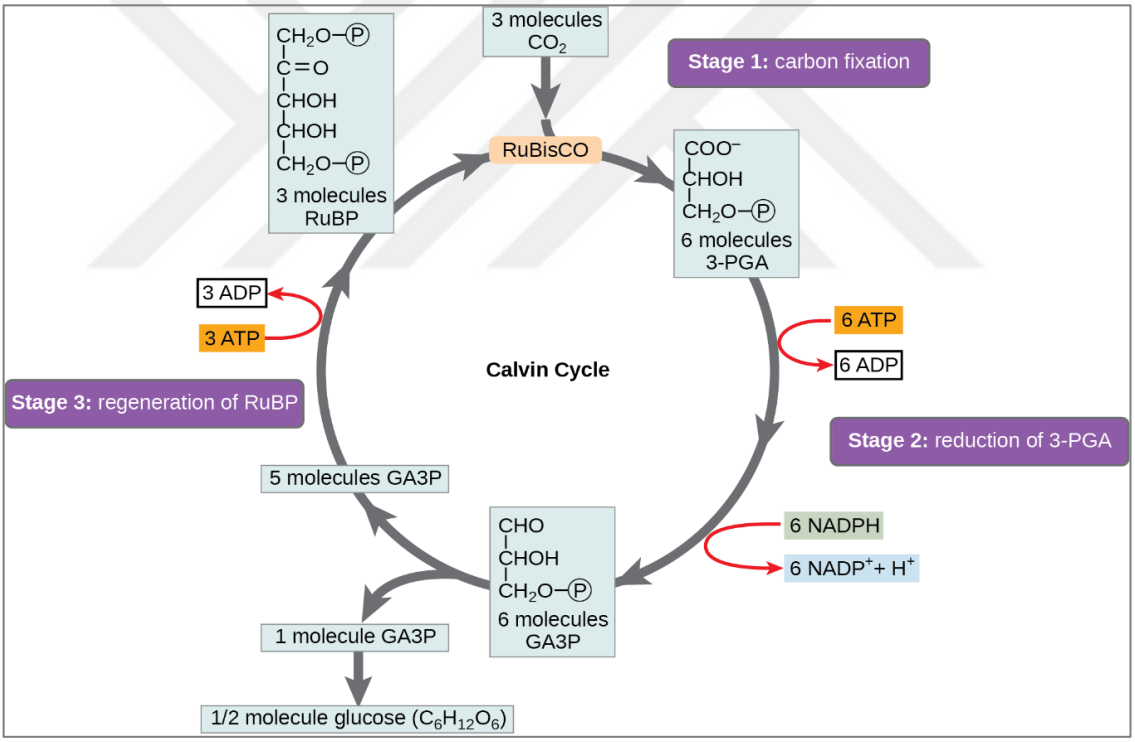


Figure 1.8. Calvin-Benson cycle in *C. reinhardtii*. The cycle consists of 3 stages and the product is GA3P (Glyceraldehyde-3-phosphate) which is further converted into glucose (sugar). 3-PGA: 3-phosphoglycerte, RuBP: ribulose 1,5-bisphosphate (38).

1.2 Biotechnological Importance

At the present, Microalgae are known as an exceedingly rich source with high photosynthetic efficiency that allowed them to be used in nearly all fields of industry including food supplements and animal feeding, medical fields and cosmetics, and as renewable economical source of biofuels (39). With about 35.000 tons per year estimated production of microalgae biomass, China is the world's largest producer of microalgae biomass in the market as it accounts for two-thirds of the global production (40).

Microalgae are an attractive long-term source of food and compounds with improved nutritional and functional qualities. Different high-value products have been extracted and commercialized from microalgae such as pigments (such as Carotenoids and Astaxanthin), different fatty acids including omega-3, and antioxidants. Furthermore, other valuable products include various energy sources (like sustainable biofuel and biodiesel), recombinant proteins and animal feed. Nevertheless, the nutrition sector appears to profit more from microalgae biotechnology (41).

Among the highly valuable compounds produced by microalgae; carotenoids and more specifically β-carotene which was the first product commercially manufactured in the 1980s from *Dunaliella salina* (a green unicellular microalgae) (42). β-carotene is a valuable pigment with antioxidant and anticancer benefits for human body, it is also a precursor of vitamin A (43). The red xanthophyll Astaxanthin is also a carotenoid which was first identified in 1938 and employed as an aquaculture pigment. Astaxanthin is renowned for its health benefits due to having an outstanding antioxidant activity that is more than 100-fold stronger than other carotenoids and vitamins (44).

Furthermore, microalgae are known to be a valuable source of lipids and fatty acids that are known for their health benefits for humans (e.g. omega 3, omega 6, and Linoleic acid). Fatty acids can be administered as supplements for supporting the cardiovascular system and the prevention of hypertension, arrhythmia, and thrombosis (45).

Nowadays, microalgae are gaining growing interest as a sustainable source of liquid biofuels due to their high output since microalgae are capable of accumulating a significant amount of oil within their cells as well as having a fast growth, another advantage of using microalgae in biofuel is the year-round harvestability and the easier cultivation conditions (46). Unfortunately, due to the high expense of the cultivation and harvesting techniques utilized for biofuel production from algae it has not been commercialized yet, research is still ongoing for optimizing the cultivation and harvesting on a commercial and industrial size.

1.3 *Chlamydomonas Reinhardtii*: Model Organism

The motile unicellular green microalgae *Chlamydomonas reinhardtii*, named after the botanist Ludwig Reinhardt, was first discovered in 1888 by Dangeard (47). *C. reinhardtii* is one of the first algae species to be examined and modified as it is considered a model organism because of its fully sequenced nuclear, chloroplast and mitochondrial genomes and the ability of genetic manipulation, fast growth and ease of isolation (48). Furthermore, another distinguishing characteristic of *C. reinhardtii* is its ability to grow exclusively on light and it is known as photoautotrophic growth, and also completely in the dark on acetate (heterotrophic growth), this feature, along with the ongoing and expanding genome studies, enables us to conduct in-depth analysis of genes and proteins involved in the photosynthetic or respiratory, their function functions and evolution, as well as to investigate how environmental conditions and individual mutations affect the gene expression (49).

Full taxonomy of *C. reinhardtii* is listed below (50):

Kingdom	Plantae
Subkingdom	Viridiplantae
Division	Chlorophyta
Subdivision	Chlorophytina
Class	Chlorophyceae
Order	Chlamydomonadales
Family	Chlamydomonadaceae
Genus	Chlamydomonas
Species	*Chlamydomonas reinhardtii* (Dangeard, 1888)

1.3.1 Biology

C. reinhardtii is a motile green microalga that belongs to Chlorophyta group of microalgae. This microalga is characterized by two mammalian-like flagella that are responsible for its movement toward the optimal light conditions and mating through the sexual reproduction (51). The structure of *C. reinhardtii* illustrated in figure 1.9.a shows the chloroplast, in which photosynthesis occurs, as it takes up the highest portion of microalgae's cell volume, two flagella (cilia) used for motility and mating, the nucleus located in the center, along with the other components plasma membrane, Golgi body, eyespot, starch grains and the two motile cilia.

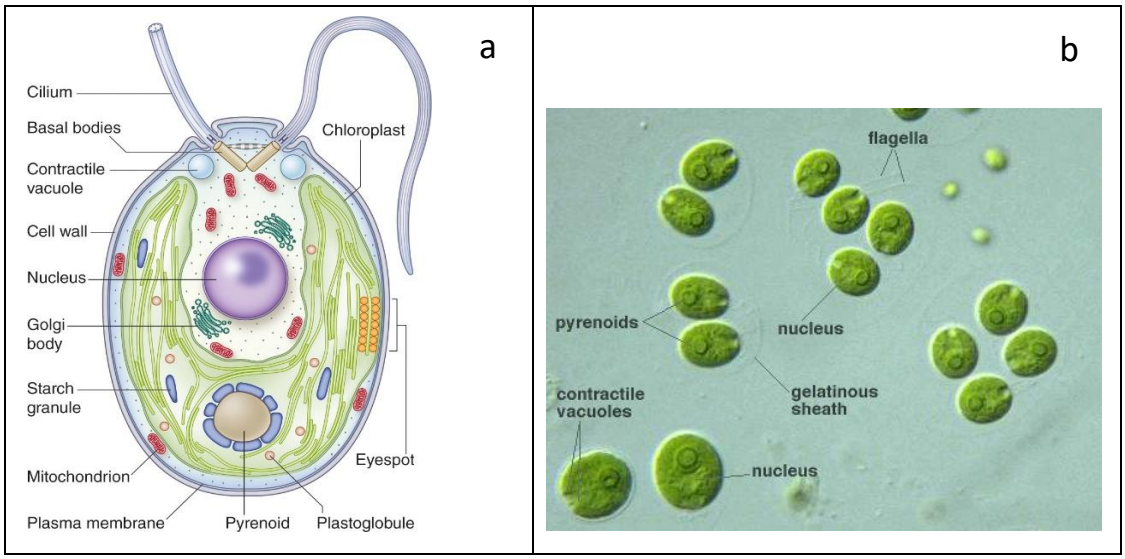


Figure 1.9. Chlamydomonas reinhardtii a) morphology (52), and b) microscopic view (53).

1.3.2 Life Cycle

The growth and reproduction in *C. reinhardtii* occur in two states as the following:

1. Vegetative growth (Asexual reproduction): is the main way for reproduction in *C. reinhardtii* and it takes place when the growth conditions are favorable. *C. reinhardtii* has the advantage of being fast growing as it can double its cell count in about 8 hours and produce up to sixteen daughter cells (54).
2. Sexual reproduction: when placed in unfavorable conditions like lack of nutrients, high salinity or low temperature, *C. reinhardtii* switches its reproduction to sexual reproduction through the production of zygotes, this process allows this microalgae to do several mutations to produce a new generation of microalgae that is more resistant and adaptive in order to survive (55). Figure 1.10 illustrates both the Asexual and sexual reproduction of *C. reinhardtii*.

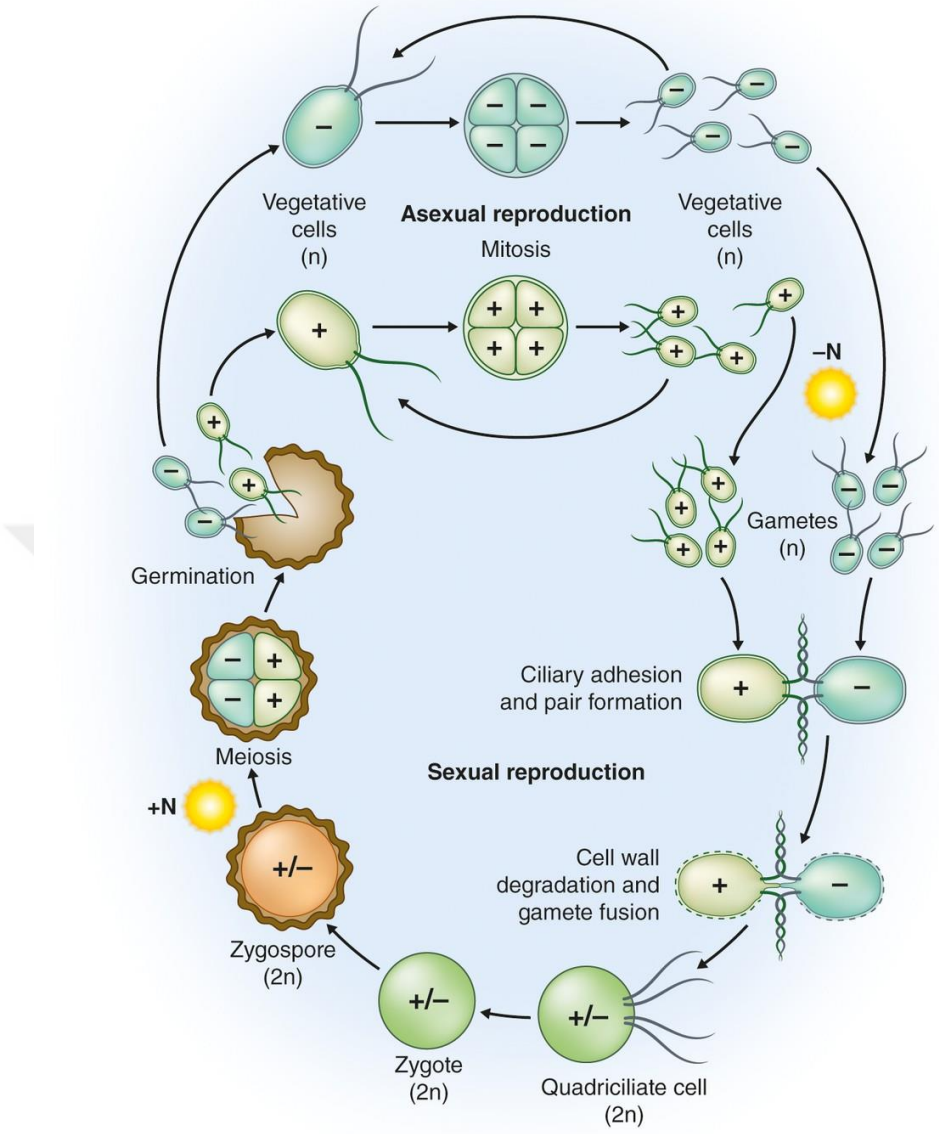


Figure 1.10. Sexual and Asexual reproduction in *C. reinhardtii* (52). In case of lack of nutrition (for example nitrogen deprivation) gametes of opposite mating type fuse together by the flagella to produce a Zygote which then, when the nitrogen is back normal, undergoes meiosis and germination (52). The asexual reproduction, under favorable conditions, consists of long Growth phase followed by DNA replication and mitosis (56).

1.3.3 Benefits and Biotechnological Importance

C. reinhardtii is known for its added nutritional value and health benefits despite its rare use as food additive. The protein content of *C. reinhardtii* is about 46.9% of dry weight, this amount is comparable to protein levels in other microalgae species like *Spirulina* and *Chlorella* that are used in the market as they are considered superfood because of their high nutritional value (57).

Furthermore, consuming *C. reinhardtii* in the diet has proven to reduce gastrointestinal symptoms associated with IBS (Irritable bowel syndrome) such as bloating and diarrhea and abdominal discomfort without negatively affecting the intestinal microbial composition (58). This highly valuable microalgae could be used in future as dry weight supplements for its health benefits and safety in terms of mutagenicity or toxicity (59).

1.4 Algae Cultivation

1.4.1 Bioreactor System

When produced in large quantities, microalgae are more efficient than agricultural plants and can be grown in non-arable ground with little or no freshwater input, in either seawater or even wastewater. However, not all geographical locations are sufficiently suitable for large scale algae crop development (22).

When cultivation algae on a large scale there are often two culturing systems: opened systems and closed systems. The opened culturing systems are mainly outdoor cultivation of algae under sunlight and natural water, this system has less cost for large scale which makes it the easiest way for large scale cultivation of algae. However, it is subjected to many limitations such as the geographical changes, variations in freshwater nutrient content and evaporation level which may cause changes in salinity levels (60). On the other hand, closed culturing systems, also called photobioreactors, insure the cultivation of algae under controlled light irradiance of different sources (such as Fluorescent, Halogen, or LED lights) along

with culturing media (bioreactors) with measured nutrients and salinity levels (61). Unlike the open pounds, closed systems insure a full light penetration to the culture medium along with a lower space required for effective cultivation which makes it ideal for studies and optimizing the productivity of algae (62). Table 1.1 demonstrates the main differences between open system and closed system.

Table.1.1 Comparison between open system and closed system for algae industrial production (62); (63).

comparison	Open system	Closed system
cost	Low cost	Higher cost
contamination	High risk of contamination	Lower risk of contamination
Light utility effectiveness	Sub-optimal utility, light penetration is limited in high density cultures.	Optimal light usage (culture mixing and control).
Source of light	Sunlight	Artificial light source (such as: LED, fluorescence).
Growth medium	Natural water	Controlled bioreactors
CO_2 level	CO_2 levels depends on whether (variable)	Constant CO_2 and gas supply (controlled)

1.4.2 Light

Increased biomass production, up to commercial scale, is one of the primary obstacles to realizing microalgae's full potential in the market and the most important limiting element for microalgae growth and cultivation is light (64). As known for algae growth, the most important environmental factors impacting algae development are the light and temperature. Furthermore, photosynthesis, the most significant step in the growth of autotrophic microorganisms, rely on light as a source of energy. As reported by de Mooij et al., (65), efficiency of photosynthesis is observed when microalgae are exposed to low light levels. On the other hand, a

high photosynthetic efficiency is challenging under high light intensities (such as strong sunshine) because the cells collect more light energy than their capacity to convert them into biochemical energy. As a result, microalgae release some of the excess light that was absorbed in the form of heat and photodamage may occur (65). Furthermore, microalgae continue to grow and increase its density under the effect of light until it reaches a light saturation point where the growth rate increase will be no longer observed (66). If all photons produced form the light source were absorbed by the photosynthetic system of microalgae, the maximum photosynthetic efficiency and grow will occur. To reach this, the light source spectrum should completely match the photosynthetically active spectrum of the algae species studied (67).

Sunlight is the natural and most cost-effective light source for outdoor cultivation of energy. This sustainable light source has several drawbacks due to the difficulty of controlling it, geographical variations and fluctuating radiation that might be either low photosynthetic efficiency or a damaging radiation to algae cells (68,69).

In indoor closed systems, artificial lighting provides the advantage of controlled and increased productivity of cultivated microalgae and thus overcomes the drawbacks of the natural light. However, it comes with a greater production cost as the high production yield and density cultures requires that the cells receive the optimally light energy necessary for its growth throughout all its growing cycle which drives the researchers to continuously seek new methods and technologies with reduced costs, thereby increasing the production rate (70).

1.4.3 LED Light

It is known that different species of microalgae respond differently to the light they receive depending on many factors including their pigment content. But for all microalgae, a balanced spectrum of wavelengths is essential for the growth and stability. Thus, when used appropriately, LED lights have the potential to not only improve the quantity of the microalgae biomass, but also the quality of the production yield (28).

LED light refers to Light Emitting Diodes, it works by transferring the electricity passed through the semiconducting diode (shown in figure 1.11) into light of a narrow spectrum. The first study regarding LED lights was published in 1907 about infra-red LED light, however, it was not used for many years until Nick holonyak jr (1962) created the first usable LED light (red LED) (71).

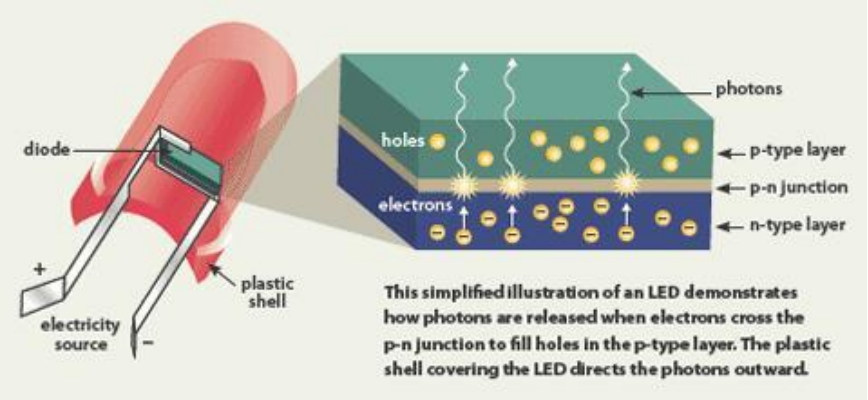


Figure 1.11. A Simplified illustration of LED lighting system. The diode releases light photons as the electricity passes through its layers (72).

Conventional lights like fluorescent and halogen bulbs are known to consume a tremendous amount of electricity with just marginally positive effects on the photosynthetic activity of algae. On the other hand, here are some benefits of LED lighting: 1) more cost-effective on long term, 2) more energy-efficient due to the lower generation of heat, 3) more durable with longer working life, 4) and provides a greater degree of control and stability (73).

In addition, LED light provides a narrow peak light of the most important wavelengths for photoautotrophs growth (like blue and red lights) unlike the full spectrum nature light which includes different wavelengths (like ultraviolet and infrared lights of wavelengths less than 380nm, and higher than 700 nm respectively) that are beyond the photosynthetic active radiation and might results in damage to algae and plants (74). Figure 1.12 shows the different light spectrum of LED and natural light.

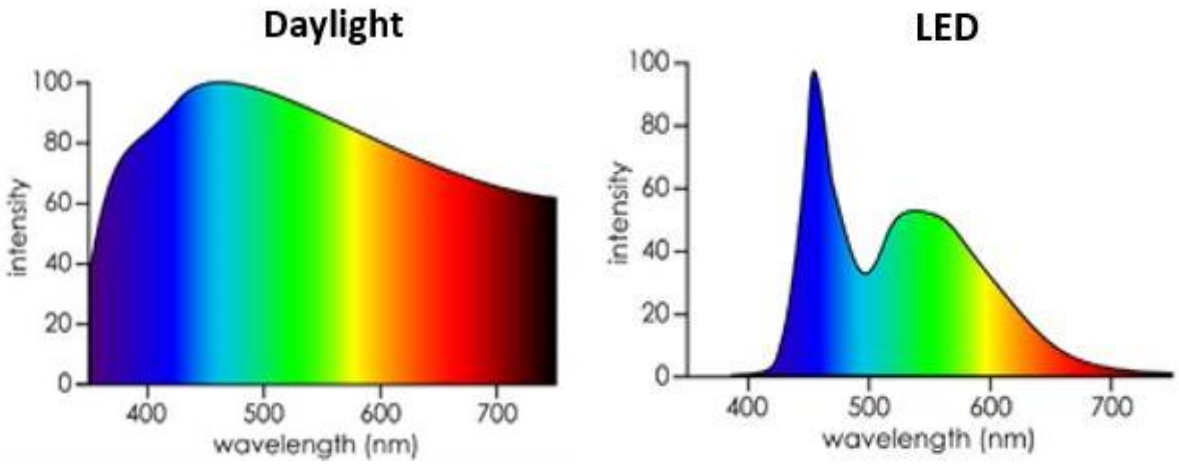


Figure 1.12. Difference in light spectrum between daylight (natural light/ full spectrum) and LED light (peak wavelengths of PAR) (75).

Nowadays, LED lights are gaining a wide interest of use ranging from entertainment in houses, to researches and cultivation, and even in space with many different uses and applications. Especially in laboratory studies and indoor cultivation of algae cultures, LED lights have proven to be a very efficient lightening system in enhancing the biomass and yield of algae products.

1.4.4 Red and Blue Light

Numerous scientific researches are published regarding the effect of LED light on the growth of algae and production yield of specific metabolite, it is certain that the effect of specific wavelengths of LED light may produce different effects depending on species type and cultivation conditions. This means that there is not a single wavelength that is the best for all algal species or for all purposes.

Previous studies concluded that blue and red wavelengths are the primary wavelengths controlling the cell division and photosynthesis in green microalgae because of the wavelength compatibility with the absorption of chlorophyll and other light absorbing pigments (76). However, cultivation of algae under either red or blue light alone has shown to be damaging on the long-term and therefore the

combination of red and blue light will compensate the damaging effect of using monochromatic light (77).

Blue light fractions are required to produce higher growth in green algae, this is because it is essential in activation of carotenoids which is vital in building PSII and its related polypeptides like LHCI. Also, blue light absorption by chlorophyll a pigment stimulates the production of phycobiliproteins and thus enhance the productivity (78).

Many studies studied the advantages of using red and blue light combinations in growth of microalgae. For instance, a research on *Nannochloropsis oculata* and *Tetraselmis chuii* (two green microalgae) showed that the combination of both red and blue lights (dichromatic light supplement) are the optimal for algae growth, and monochromatic lights (single light color) or light combinations missing these two wavelengths (red and blue) are not able to stimulate the optimal growth of algae (79).

Furthermore, a study by Oldenhof et al. (20) showed that When applying blue light (400-500nm wavelength) to *C. reinhardtii* cells at a certain growth time, the threshold cell size needed for cell division is shifted to a later time resulting in larger cells' size and greater proliferation. on the other hand, red light (600-700nm) counteracts this effect thus inducing the cell division resulting in smaller cells' size. Also, as mentioned before, the maximum absorption of chlorophyll pigments found in algae and plants is known to be at 425nm and 665nm wavelengths which represent the blue and red light respectively (80).

1.4.5 Green Light

Supplementation of green light (wavelength of 500-550nm) into the light provided for indoor cultivation of algae species was not a case of interest in the previous studies since it is known for not having a direct effect on the growth of algae and photosynthesis process and its reflected by plants and algae giving them their distinguishing color (81). However, more recent researches continuously aiming to optimize the photobioreactor lighting system for algae growth found that

even though the poorly absorbed wavelengths like green light alone are not efficient in promoting photosynthesis, but these wavelengths have a fundamental role the growth of algae and plants specially in dense cultures (82).

Green light supplementation effect on growth was firstly studied on plants. Kim et al., (83) studied the effect of adding green light to red and blue LED lights in lettuce, and found that the green light penetrates the dense plant canopy better than blue and red wavelengths and thus the addition of green light to a certain ratio (approximately 24%) promotes the plant growth rate (83). Furthermore, partial replacement of green light to blue and red wavelengths on tomato enhanced the growth and morphology (84).

As for algae, green light was less preferred for growth as compared to red and blue lights, and studies found that green microalgae photosynthetic activity was suppressed when grown under green light alone (85). However, more recent studies found that green wavelength promotes pigments production specially in dense algae cultures through its high penetration in comparison to red and blue lights (86). Furthermore, study by Kalmaoğlu (87) on *Haematococcus pluvialis* algae showed a similarity between algae and plants response to green light supplementation to red and blue combinations. A significant increase in the algae growth and chlorophyll-a content by the upregulation of gene expression indicates the fundamental role of additive green light in the growth of algae and plants (87).

When exposed to different wavelengths and under high light intensity, microalgae start to produce electron acceptors to reduce the activity of the electron transport chain. This protective mechanism is beneficial in lowering the photoinhibition and cell damage, but also results in higher accumulation of fatty acid (88). Green light was found to be effective in enhancing this photoprotection process and production of CO_2-derived fatty acids (89). However, additional studies regarding the harvesting and effect of green light supplementation on the growth of algae and production of secondary metabolites is required.

2. AIM AND SCOPE OF THE STUDY

There are 2 aims in the present study to characterize the optimal LED light combination to enhance the growth rate of *C. reinhardtii*. The first aim is to test and compare monochromatic red and blue, different ratio of dichromatic red and blue combinations, and white light effects on the growth rate of *C. reinhardtii*. The second aim is to study if there is an additive effect of green LED light combinations on the growth of *C. reinhardtii*. This effect is studied in conjunction with red and blue LED light combinations aiming to establish the ideal (optimal) light combination for *C. reinhardtii* biomass production. The data and analysis reported at the conclusion of this study may be utilized in addition to the published data in designing the optimal cultivation system for large-scale industrial production of *C. reinhardtii* and, consequently, the highest yield of high-value metabolites.

The experimental setup used in this study is divided into two parts with the following objectives:

1. The first part aims to define the optimal growth and biomass production of *C. reinhardtii* under the effect of red and blue LED light separately and in multiple-ratio combination. our hypothesis is to test if a specific combination of both red and blue light will result in a higher growth of *C. reinhardtii* than the white light or monochromatic red or blue lights.
2. The second part is applying different ratios of green wavelength light in addition to the optimal light condition conducted in the first part of the experiment in a percentage-based supplementation to investigate its effect on the growth kinetics of *C. reinhardtii* as well as targeting to optimize the light condition to produce the highest growth of this microalgae species.

3. MATERIALS AND METHODS

The experiments were carried out to characterize the effect of different LED light illumination conditions on the growth kinetics and chlorophyll production of *C. reinhardtii*. each experiment treatment was carried out as 3 biological replicates to ensure the accuracy of the results. The resources and research methodologies used throughout the thesis are described in the sections that follows.

3.1 Microalgae Strain and Cultivation Conditions

Freshwater *C. reinhardtii* stock culture was obtained from UTEX algae culture collection Massachusetts, USA (UTEX number: 89). Stock cultures were maintained in 250 ml Erlenmeyer flasks with 200 ml of BG-11 (table 3.1). BG-11 medium was prepared by adding its components to distilled water with continuous stir mixing. The pH level was adjusted to 7.2-7.3 before autoclaving for 1 hour.

Table 3.1. BG-11 medium chemical composition and concentration (90).

#. Component	Final concentration, mM
1. $NaNO_3$ (Merck 1.06535)	17.6 mM
2. K_2HPO_4 (Merck 1.05099)	0.23 mM
3. $MgSO_4.7H_2O$ (Merck 1.05886)	0.3 mM
4. $CaCl_2.2H_2O$ (Merck 1.02382)	0.24 mM
5. Citric acid.H_2O (Sigma 251275)	0.031 mM
6. Ferric ammonium citrate (Sigma F-5879)	0.021 mM
7. $Na_2EDTA.2H_2O$ (Sigma E5184)	0.0027 mM
8. Na_2CO_3 (Merck 1.06392)	0.19 mM
9. BG-11 Trace metals solution (1ml/L)	
• For agar media only: Sodium Thiosulfate Pentahydrate	1 mM

After inoculating the cultures into the erlenmeyer flasks containing the BG-11 media with initial concentration of 0.05 OD value, the flasks were covered with parafilm and transferred to the photobioreactor. Sterile air filters were used to prevent the contamination and insure a consistent air flow into the cultures.

Cultures were kept under continuous air flow enriched with 2% (v/v) CO_2 and a constant temperature of 25 ± 0.5°c, these parameters were measured continuously ensuring their stability to eliminate their effect on the growth of microalgae species during the study. Furthermore, all the sterilization precautions were taken to prevent any contamination of the cultures and to minimize any external factors altering the study. Picture 3.1 shows solid and liquid culture of *C. reinhardtii*.

Picture 3.1. Solid and liquid stock cultures of *C. reinhardtii*.

3.2 LED Light Condition Setup

The study is divided into two parts using the same conditions of the stock cultures with various light combinations in order to evaluate the effect of green LED light illumination in combination with red and blue light on the growth of *C. reinhardtii* algae culture.

Samsung 50/50 RGB LED strips were located 2cm under the flasks with a diffuser sheet to insure uniform and equal light distribution to each sample, the light intensity was measured using OHSP-350 portable LED spectrometer (HOPOOCOLOR, China) and the light quality and quantity were adjusted using Arduino based computer card and software program. Throughout the study, the cultures were kept away from other light sources (such as daylight or room light) effects by covering the windows and keeping the room dark. Additionally, black plexiglas were used to cover each light compartment. Also, cultures were regularly shaken 2-3 times per day to prevent sedimentation of the algae.

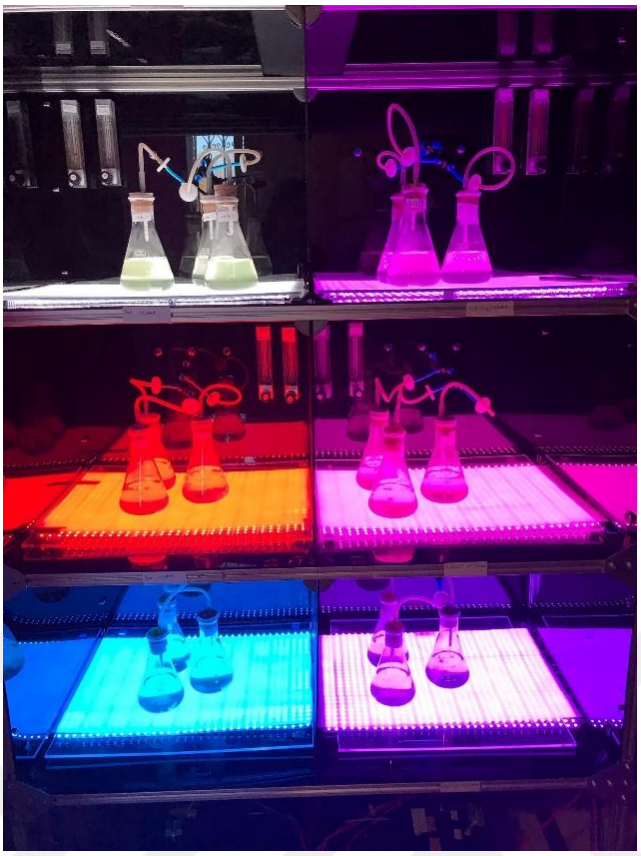
The two experiment parts made in this study are further discussed in the following section.

3.2.1 Red and Blue Lights Experiment

Combinations of red and blue lights reported to enhance the growth of *C. reinhardtii* more than monochromatic red or blue light. Six experimental treatments were used to study the effect of monochromatic and dichromatic red and blue LED lights in different rations on the growth of *C. reinhardtii* and find the optimum light condition. The cultures were illuminated with total 40 $\mu mol/m^2.s$ light intensity, for 16h light:8h dark. Samples were taken regularly for measuring OD value (every day) and chlorophyll concentration (every 2 days) for each sample. Furthermore, dry weight samples were taken at the end of the experiment. Picture 3.2 shows the LED light compartment set up with samples in it. The experimental groups and light intensity distribution are shown in table 3.2.

Table 3.2. Light intensity distribution in red and blue light experimental treatments.

Light treatment	Content	Light intensity
White	illuminated with white light	40 μmol/m^2.s.
Red	illuminated with monochromatic red light only.	40 μmol/m^2.s.
Blue	illuminated with only monochromatic blue light.	40 μmol/m^2.s.
R1/B1	illuminated with dichromatic Red and blue lights of equal ratios.	20:20 μmol/m^2.s respectively
R2/B1	illuminated with red and blue light combinations with higher red-light ratio.	27:13 μmol/m^2.s respectively.
R1/B2	illuminated with red and blue dichromatic light with higher blue-light ratio.	13;27 μmol/m^2.s respectively.



Picture 3.2. LED light system setup. each sample was placed in a specifically located place throughout the experiment.

3.2.2 Green Light Supplementation Experiment

In the experimental groups, green light was supplemented as a fraction of both red and blue lights (ratio 1:2 respectively) with Total light intensity of 40 μmol/m^2.s (same as the first experiment). The chromatic diagram of the experimental groups is illustrated in figure 3.1. The light intensity distribution for the experimental groups were as described in table 3.3.

Table 3.3. Green light supplementation light intensity distribution and percentage of green light in experimental treatments.

Light treatment	Content	Light intensity	% of green light
Control	illuminated with only blue and red lights (no green light).	27:13:0 μmol/m^2.s respectively	0 %
G-1	illuminated with blue, red, and green LED lights. 3 μmol/m^2.s green light increased in expense of 2:1 μmol/m^2.s blue: red.	25:12:3 μmol/m^2.s respectively	7.5%
G-2	illuminated with blue, red, and green. 6 μmol/m^2.s green light.	23:11:6 μmol/m^2.s respectively	15%
G-3	illuminated of blue, red, and green LED lights. Green light reached 9 μmol/m^2.s.	21:10:9 μmol/m^2.s respectively.	22.5%
G-4	blue, red, and green lights, green light ratio in higher than red light. 12 μmol/m^2.s	19:9:12 μmol/m^2.s respectively.	30%
G-5	green light ratio in this group is the greatest compared to the other groups. 15 μmol/m^2.s	17:8:15 μmol/m^2.s respectively.	37.5%

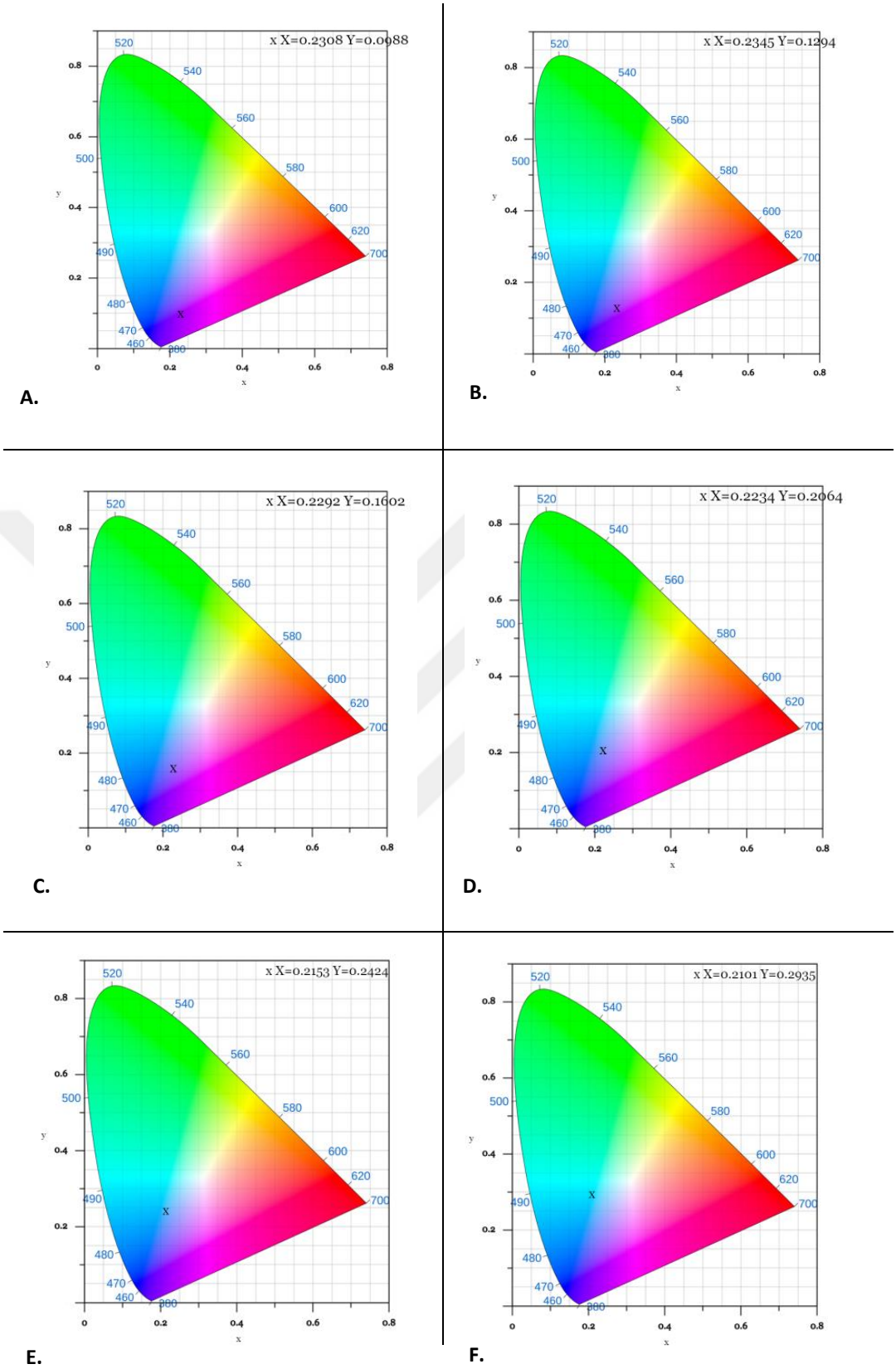


Figure 3.1. Chromaticity diagram for green LED light supplementation experiment. a) control group where green light ratio is 0% of total light intensity applied, b) Group 1 with 7.5% of green light, c) group 2 with 15% of green light,

d) Group 3 with 22.5% of green light, e) Group 4 with 30% of green light, f) Group 5 with 37.5% of green light (Data created using CIE 1931 web-based app (91)).

3.3 Growth Measurement:

3.3.1 Biomass Production Analysis

The biomass production of *C. reinhardtii* was measured by the optical density (OD) value at a regular interval using UV-6300PC Double Beam spectrophotometer. 2ml sample was taken was placed in 2ml glass cuvette, mixed thoroughly by pipetting, and absorption was measured at 664 nm after blanking with BG-11. In case the OD value was higher than 1 the results become unreliable due to the light scattering by the dense culture and gives false (low OD value) result. For this reason, the sample is diluted with BG-11 and re-measured. The OD values were recorded and plotted on excel graph against time and growth curve was drawn.

3.3.2 Dry Weight Measurement

The dry weight of *C. reinhardtii* samples was measured at the end of each experiment. 10 ml of each sample was taken and sieved through pre-weighed 0.45 μm membrane filter. after suction of water from the sample, the algae pallets were placed in 70°C oven overnight for drying before measurement.

3.3.3 Chlorophyll Estimation and Calculation

1 ml of well-mixed culture sample was taken and placed into 1.5 ml eppendorf tubes. The samples were centrifuged at 5000 rpm for 5 minutes. After centrifuge, the supernatant was discarded, and 1 ml methanol was added. Thorough mixing by vortex and pipetting was made to insure appropriate dissolving of the

algae pallet with the methanol. The samples were then covered with aluminum foil and incubated in refrigerator at 4°C overnight. After incubation, the samples are checked for white pallet to ensure that all the chlorophyll was fully extracted, then recentrifuged again at 5000rpm for 5 minutes. Finally, 1 ml of the sample mixture is taken into the cuvette and mixed with 1 ml methanol, OD value was measured spectrophotometrically at wavelengths 663 nm and 645 nm after blanking with methanol.

The chlorophyll concentration is then calculated according to the following equation where A_{663} is absorbance at 663 nm wavelength, and A_{645} is absorbance at 645 nm:

$$\text{Chlorophyll a } (\mu g/ml) = 12.7\ A_{663} - 2.69\ A_{645}$$

$$\text{Chlorophyll b } (\mu g/ml) = 22.9\ A_{645} - 4.68\ A_{663}$$

3.3.4 Statistical Analysis

For analyzing the effect of light on the growth of *C. reinhardtii*, post-hoc Tukey HSD one-way analysis of variance (ANOVA) was applied to test the difference between the experimental conditions results through their mean ±SD.

4. RESULTS

4.1 First Part: Red and Blue LED Lights

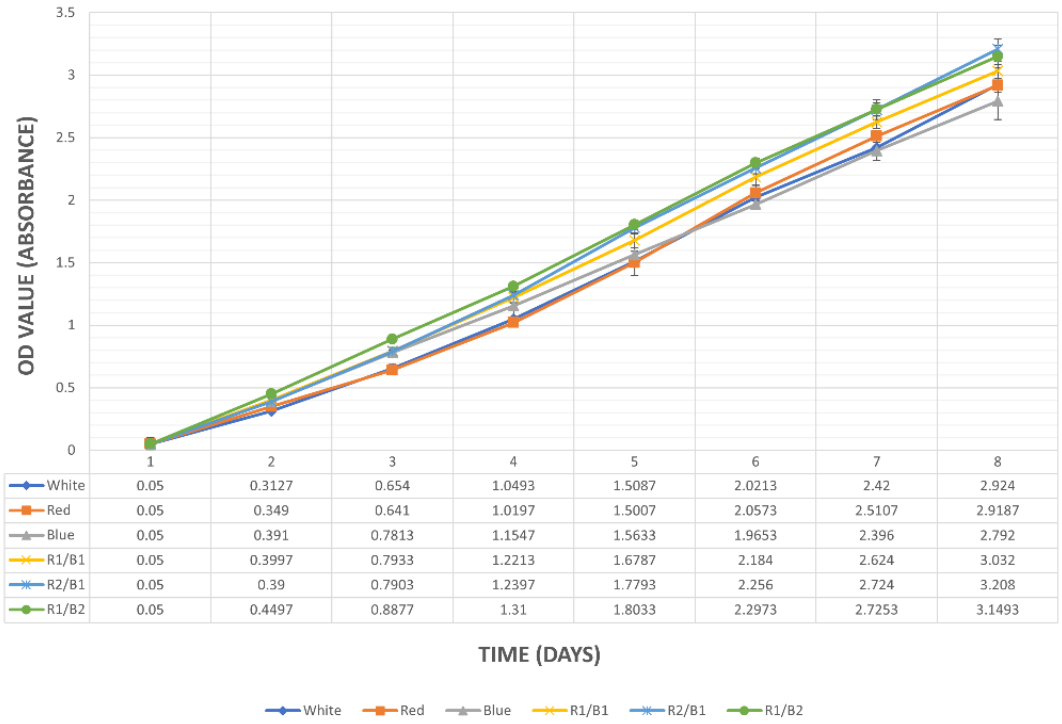
4.1.1 Growth Rate and Production Yield

The growth curves of *C. reinhardtii* under 6 different LED light conditions in 8-days cultivation period is illustrated in figure 4.1. The growth conditions where White, Red, Blue, R1/B1, R2/B1, R1/B2 LED lights. The results show that the growth rate of *C. reinhardtii* cultivated in dichromatic light is statistically higher than monochromatic light regimes. OD values in R/B (2:1) and R/B (1:2) were statistically higher than Red and Blue light while did not show statistically significant differences with white light.

The order of the growth rate from the highest to lowest was as the following:

Red: Blue (1:2) > Red: Blue (2:1) > Red: Blue (1:1) > White > Red > Blue

Nevertheless, Statistical differences where not observed when comparing dichromatic light of red/blue combinations in comparison to white light. Furthermore, no statistical differences were observed when comparing red and blue dichromatic light of the ratios 2:1 and 1:2.



	1	2	3	4	5	6	7	8
White	0.05	0.3127	0.654	1.0493	1.5087	2.0213	2.42	2.924
Red	0.05	0.349	0.641	1.0197	1.5007	2.0573	2.5107	2.9187
Blue	0.05	0.391	0.7813	1.1547	1.5633	1.9653	2.396	2.792
R1/B1	0.05	0.3997	0.7933	1.2213	1.6787	2.184	2.624	3.032
R2/B1	0.05	0.39	0.7903	1.2397	1.7793	2.256	2.724	3.208
R1/B2	0.05	0.4497	0.8877	1.31	1.8033	2.2973	2.7253	3.1493

Figure 4.1. *C. reinhardtii* growth curve under six different light conditions of monochromatic LED lights (red, blue), different ratios of dichromatic (red and blue) light, and white light. the average optical density (absorbance) at 664nm of each light condition is provided under the curve and the starting concentration for all the experimental groups was 0.05.

Table 4.1. P-value for growth rate using post-hoc Tukey HSD test ($p<0.05$*, $p<0.01$** statistically different).

light	Red	Blue	R1/B1	R2/B1	R1/B2
white	0.9000	0.6566	0.7970	0.0632	0.1828
Red		0.6878	0.7658	0.0571*	0.1669
Blue			0.1415	0.0051**	0.0156*
R1/B1				0.3993	0.7424
R2/B1					0.9000

As for the biomass production yield (measured as dry weight/10mL sample), the results show the following order from highest to lowest biomass yield:

R1/B2 > R2/B1= R1/B1 > White > Red > Blue

These results support the growth rate results in showing the best LED light conditions for the growth and, more likely, yield production of *C. reinhardtii*. Nevertheless, none of the samples showed statistical differences (table 4.2).

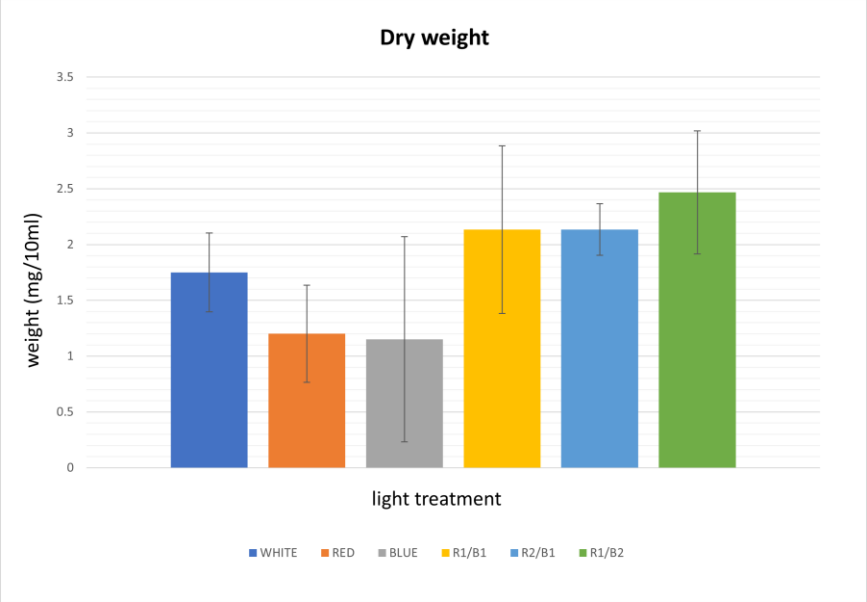


Figure 4.2. Measurements of dry weight of 10 ml of each sample taken at the end of the experiment.

Table 4.2. P-value for dry weight using post-hoc Tukey HSD test ($p<0.05$*, $p<0.01$** statistically different).

light	Red	Blue	R1/B1	R2/B1	R1/B2
white	0.8768	0.8790	0.9000	0.9000	0.7130
Red		0.9000	0.3942	0.3942	0.1486
Blue			0.4515	0.4515	0.1959
R1/B1				0.9000	0.9000
R2/B1					0.9000

4.1.2 Cell Size

Pictures of the cells were taken by a camera mounted on the top of a light microscope and the cell size was measured at the end of the experiment using image analysis system. The average of cell size (μm) for each light treatment is shown in table 4.3 along with standard deviation. Blue light showed the highest cell size, followed by other light treatments in the following arrangement with red light showing the smallest cell size:

Blue > R1/B2 > White > R1/B1 > R2/B1 > Red

Table 4.3. Cell size (in μm) of *C. reinhardtii* grown under 6 light conditions at the end of the experiment.

Light treatment	White	Red	Blue	R1/B1	R2/B1	R1/B2
Average (μm)	3.0932	2.7283	3.2621	2.9553	2.8931	3.1236
Standard deviation	±0.0753	±0.0898	±0.1090	±0.2597	±0.1041	±0.0741

4.1.3 Chlorophyll Concentration

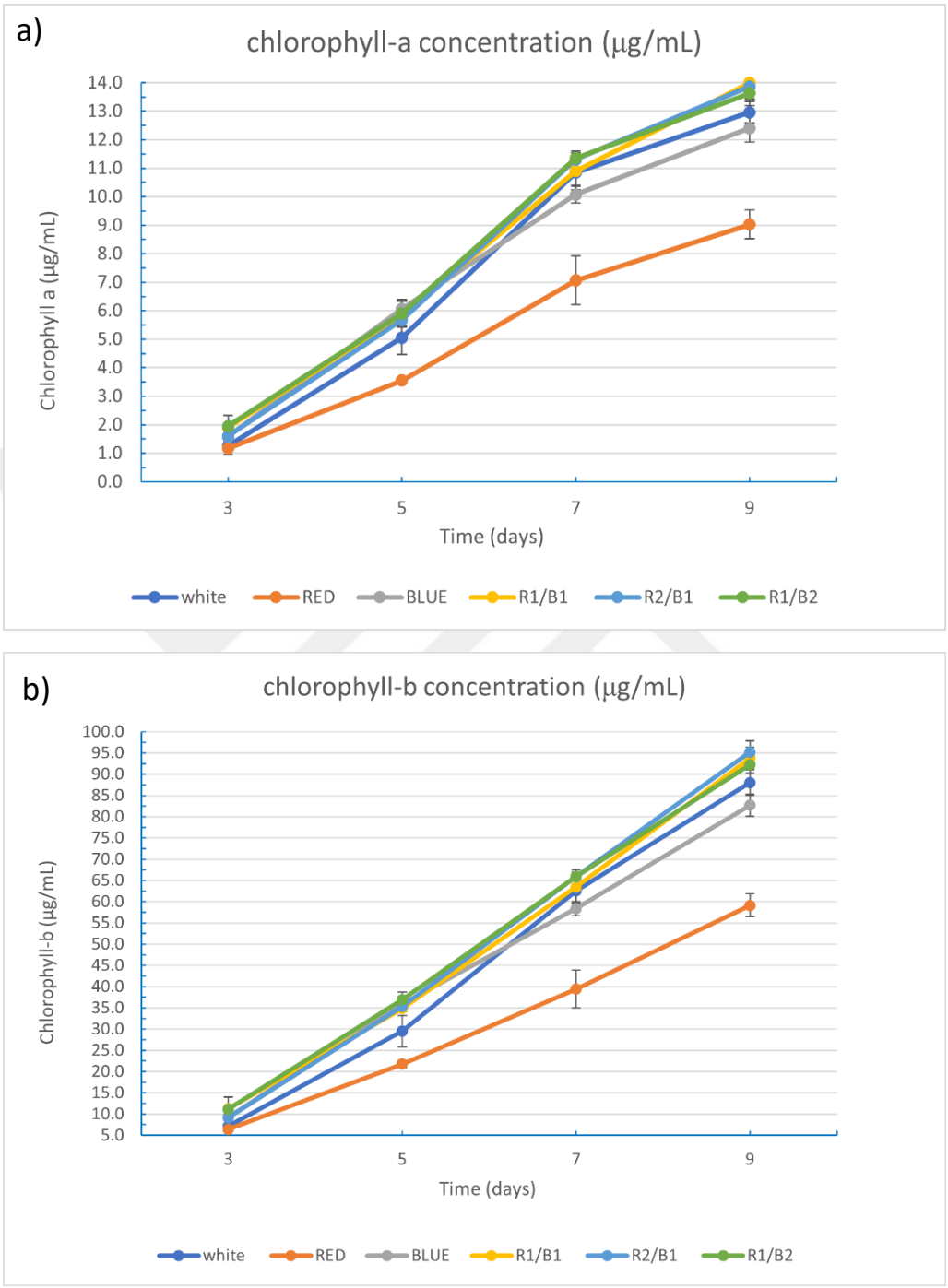


Figure 4.3. Chlorophyll measurements in *C. reinhardtii* cultivated under 6 different LED light conditions. a) chlorophyll-a and b) chlorophyll-b.

Table 4.4. P-value for chlorophyll-a content using post-hoc Tukey HSD test ($p<0.05$*, $p<0.01$** statistically different).

light	Red	Blue	R1/B1	R2/B1	R1/B2
white	0.0010**	0.6666	0.1566	0.2539	0.5341
Red		0.0010**	0.0010**	0.0010**	0.0010**
Blue			0.0081**	0.0143*	0.0410*
R1/B1				0.9000	0.8690
R2/B1					0.9000

Referring to figure 4.3 and table 4.4, statistically higher chlorophyll-a content is seen in all dichromatic light combinations compared to monochromatic blue and red LED lights. However, no statistical differences when comparing different combinations of red and blue light as well as when comparing dichromatic LED lights to white light despite of the higher chlorophyll content. These results are consistent with the growth rate and dry weight results except that here, the lowest chlorophyll content was observed under Red LED light.

R1/B1 > R2/B1 > R1/B2 > White > Blue > Red

4.2 Green Light Supplementation Experiment

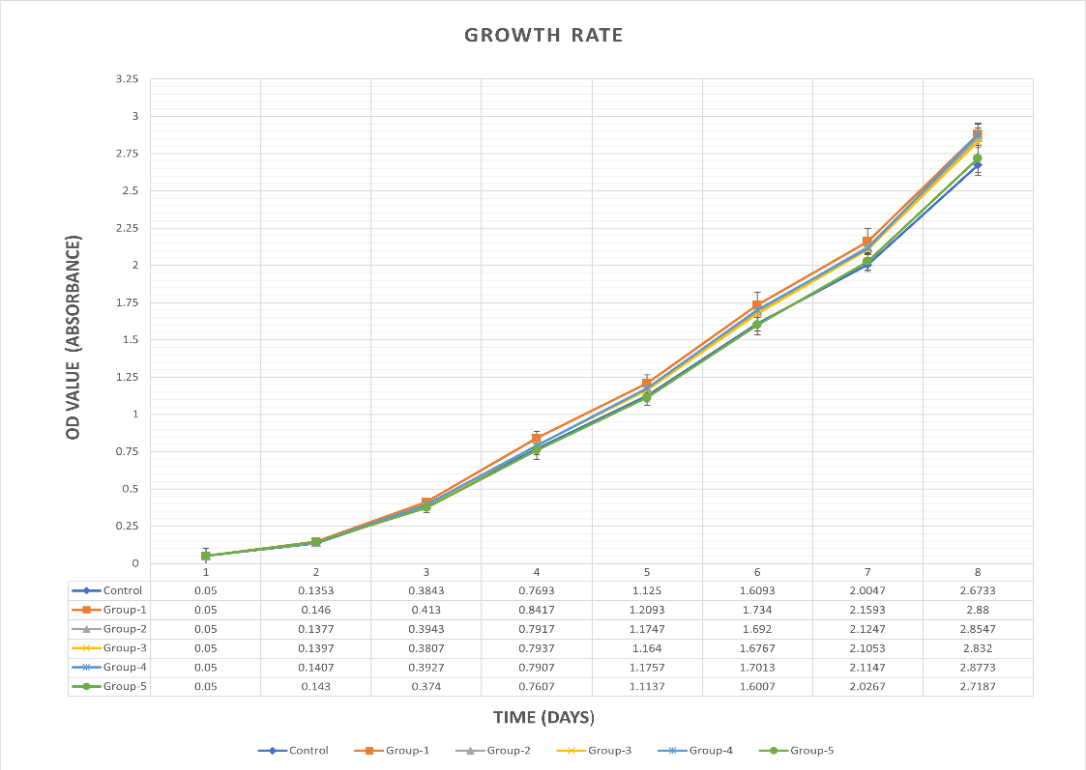
4.2.1 Growth Rate

The second part of the experiment is supplementing green LED light in different ratios aiming to study its effect on the growth rate and chlorophyll levels in *C. reinhardtii*. The control group in this experiment is R1/B2 LED light from the first part of the experiment as it has proven to be the best growing condition of the other LED light conditions tested on *C. reinhardtii*. Green LED light was supplemented in ratios 3, 6, 9, 12 and 15 μmol/m^2.s as a fraction of red and blue LED lights (subtracting 1:2 μmol/m^2.s of red and blue lights respectively).

The growth curve in figure 4.4 shows that the highest growth rate was under the 3μmol/m^2.s green light supplementation and the lowest growth rate was observed in control group (only red and blue). Interestingly, the 15 μmol/m^2.s supplemented green light (highest ratio of green light) showed a lower growth compared to the other green light supplemented groups and yet higher growth than control group.

Group-1 > Group-4 > Group-2 > Group-3 > Group-5 > Control

The order of the growth rate of different ratios of supplemented green light suggests a dose-dependent effect of it on the growth of *C. reinhardtii*. Nevertheless, none of the results showed statistical differences from each other despite the differences in the growth rate (table 4.5).



	1	2	3	4	5	6	7	8
Control	0.05	0.1353	0.3843	0.7693	1.125	1.6093	2.0047	2.6733
Group-1	0.05	0.146	0.413	0.8417	1.2093	1.734	2.1593	2.88
Group-2	0.05	0.1377	0.3943	0.7917	1.1747	1.692	2.1247	2.8547
Group-3	0.05	0.1397	0.3807	0.7937	1.164	1.6767	2.1053	2.832
Group-4	0.05	0.1407	0.3927	0.7907	1.1757	1.7013	2.1147	2.8773
Group-5	0.05	0.143	0.374	0.7607	1.1137	1.6007	2.0267	2.7187

Figure 4.4. Growth of *C. reihardtii* under green light supplementation. The control group is red and blue light only (ratio 1:2 respectively), group-1 is supplemented with 3µmol/m^2.s green light as a fraction of red and blue light. Group-2 supplemented with 6 µmol/m^2.s green light, Group-3 contained 9 µmol/m^2 .s, Group-4 12 µmol/m^2 .s green light, and Group-5 with 15 µmol/m^2 .s green light. All samples started with 0.05 culture concentration.

Table 4.5. P-value for growth rate using post-hoc Tukey HSD test ($p<0.05$*, $p<0.01$** statistically different).

light	Group-1	Group-2	Group-3	Group-4	Group-5
Control	0.0746	0.1379	0.2317	0.0797	0.9000
Group-1		0.9000	0.9000	0.9000	0.2185
Group-2			0.9000	0.9000	0.3709
Group-3				0.9000	0.5458
Group-4					0.2317

Measurements of the dry weight (figure 4.5) demonstrated an interestingly different results than the growth rate. however, these results were statistically indifferent. Dry weight order was the following:

Group-1 > Group-4 > Group-5 > Group-3 > Group-2 > Control

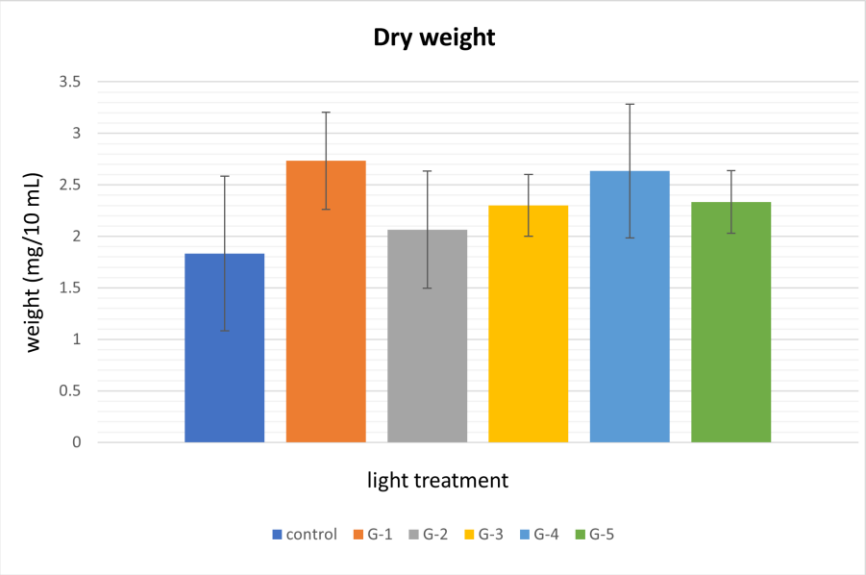


Figure 4.5. Measurements of dry weight of 10 ml of each sample taken at the end of the experiment.

Table 4.6. P-value for dry weight using post-hoc Tukey HSD test ($p<0.05$*, $p<0.001$** statistically different).

light	Group-1	Group-2	Group-3	Group-4	Group-5
Control	0.3672	0.9000	0.8775	0.4834	0.8381
Group-1		0.6410	0.9000	0.9000	0.9000
Group-2			0.9000	0.7593	0.9000
Group-3				0.9000	0.9000
Group-4					0.9000

4.2.2 Chlorophyll Concentration

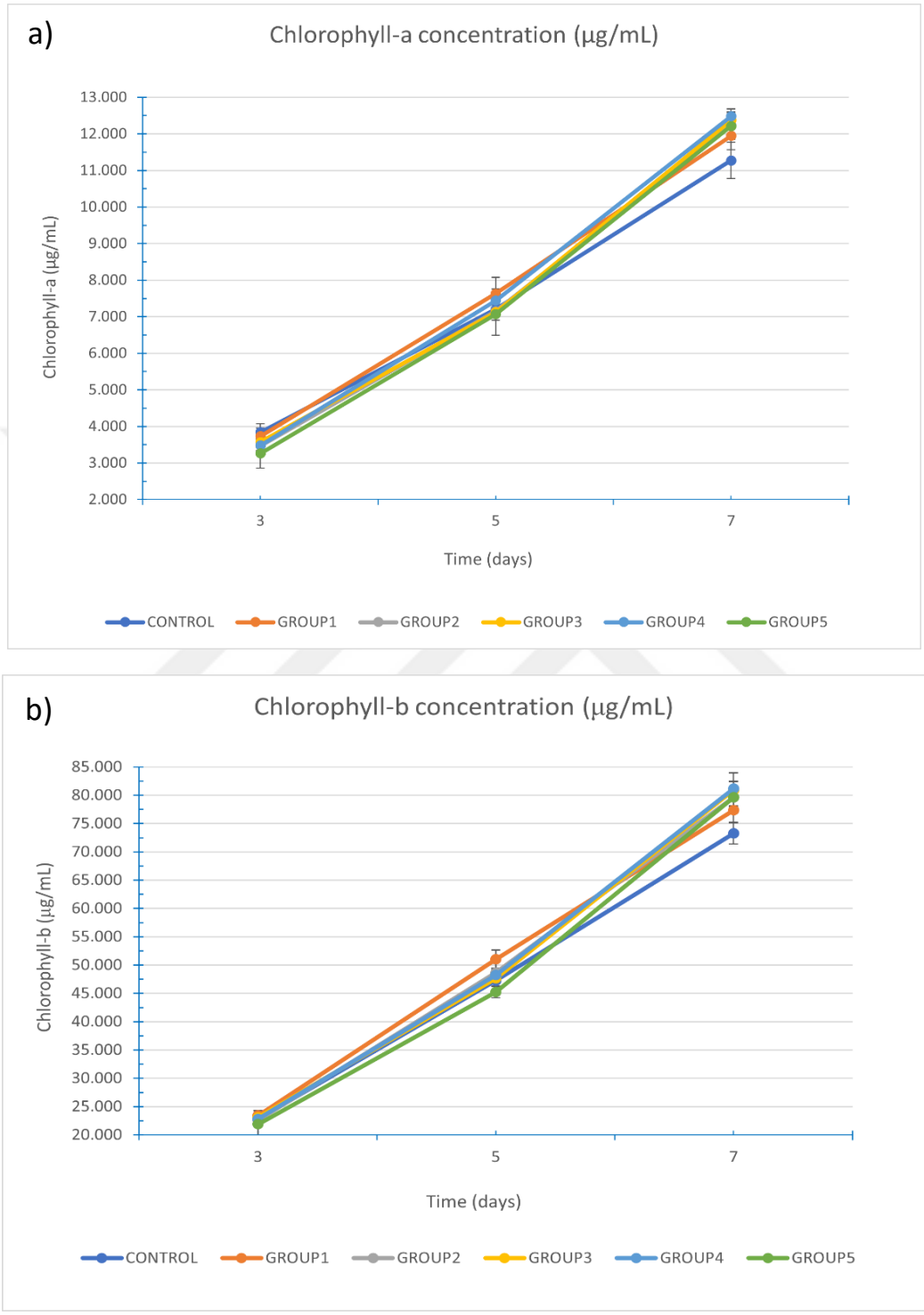


Figure 4.6. Chlorophyll concentrations in *C. reinhardtii* cultivated under different LED light conditions. a) chlorophyll-a concentration and b) chlorophyll-b. Control group consists of red and blue lights only (2:1) and other groups contains different ratios of supplemented green LED light.

Table 4.7. P-value for chlorophyll-a content using post-hoc Tukey HSD test ($p<0.05$*, $p<0.001$** statistically different).

light	Group-1	Group-2	Group-3	Group-4	Group-5
Control	0.2277	0.0475*	0.0222*	0.0108*	0.0512*
Group-1		0.9000	0.5296	0.4276	0.9000
Group-2			0.9000	0.9000	0.9000
Group-3				0.9000	0.9000
Group-4					0.9000

Both chlorophyll-a and b concentration measurements demonstrated that Control group, in which there is no green light supplementation in it, is statistically lower than other groups with green light supplementation. Results ordered as the following:

Group-4 > Group-3 > Group-2 > Group-5 > Group-1 > Control

5. DISCUSSION

Microalgae are known to for their importance in biotechnology field as a sustainable source of many valuable metabolites. Recent studies focus on the light as a critical factor effecting the growth and metabolites production in microalgae, and the use of LEDs as light source gained the highest interest because of their advantages over the conventional light sources for optimizing the growth and large-scale production of microalgae (92).

5.1 Effect of Mono and Dichromatic LED Lights on The Growth of *C. reinhardtii*

Microalgae are known to use sensory and accessory pigments to regulate their physiology and growth as a response to the light quality available for it. The growth curves of *C. reinhardtii* was studied under 6 different light conditions including monochromatic and dichromatic red and blue LED lights along with white light in an 8-days cultivation period and light intensity of 40 μmol/m^2.s aiming to investigate the optimal light condition for the growth of *C. reinhardtii*.

In this study, the results demonstrated that the highest growth rate and production yield (dry weight) were achieved when the samples were cultivated under dichromatic red/blue light illumination at ratio 1/2, while the least growth rate and yield was observed under monochromatic blue light. These results are consistent with the published data regarding the effect led light combinations on the growth of *C. reinhardtii*. For instance, He et al. (93) stated that both red and blue LED lights promotes *C. reinhardtii* growth rate and H_2 production rate, the reason of this is that stronger absorption ability to the light energy occurs under either blue or red LED light. Additionally, a study by Kim et al. (94) showed that *C. vulgaris* both cell concentration and lipid productivity increased when blue light was supplemented in the first stages of growth then switched to red light thereafter.

Furthermore, monochromatic blue light produced lowest growth rate and dry weight yet the largest cell size, this is probably because the blue light is known for

delaying the cell division and producing larger cell size in comparison to red light which accelerates the cell division (95).

It was found that combination of red and blue lights of the ratios 2:1 and 1:2 respectively both improved the growth rate of *C. reinhardtii*. However, statistical differences were not significant between the two treatments. The reason for this could be explained by the fact that blue and red light influence different parts of the photosynthetic apparatus and photoreceptors in *C. reinhardtii*. this could be explained as the following:

1) In blue dominated light combination (red1: blue2), blue light might enhance the electron transfer from PSII to PSI by activating cryptochrome photoreceptors, chlorophyll, and carotenoid pigments (78). The delay in cell division effect produced by blue light is counteracted by red light and thus higher production rate is produced by this light combination (20).

2) In red dominated light combination (red2: blue1), red light might result in acceleration of cell division through the absorption by chlorophyll pigments and lipid accumulation (96). However, the high intensity of red light was found to cause cell damage which is repaired by the addition of low amount of blue light (97).

5.2 Effect of Mono and Dichromatic Light on Chlorophyll Concentration

As for chlorophyll concentrations, despite having cultivated under the same light intensity, *C. reinhardtii* chlorophyll concentrations were significantly lower under monochromatic red or blue lights than combinations and white light ($p<0.05$).

Furthermore, red light resulted in significantly lower chlorophyll pigment in comparison to blue light. The reason for this might be that blue light was found to activate certain photoreceptors, specially phototropins, that induce pigment accumulation like chlorophyll and carotenoids (98).

5.3 Green LED Light Supplementation Effect on Growth of *C. reinhardtii*

Green light was thought to be extremely unfavorable for growing green algae. However, because it penetrates the algae culture more deeply than other wavelengths (red and blue) and has a lower potential for causing photo-saturation, light containing certain proportion of green wavelengths might be advantageous for growth of highly dense cultures and long light path (73). Furthermore, it was found that *C. reinhardtii* has light-dependent photoreceptors called rhospsins-like photoreceptors that are sensitive to green light. One of these photoreceptors specially regulates the movement of microalgae as a respond to light (photo-movement and orientation) (99). A study by Mattos et al., (100) on *Scenedesmus buija* found that supplementing green wavelength into dense cultures resulted in higher biomass production rate (8.43% growth) in comparison to red light (4.35%) in a 6 days experiment as it caused an increase in chlorophyll a and b pigments.

Another study on green microalgae *Ettlia sp*. showed a higher growth rate under green LED light than blue and red lights. The reason for this was suggested to be related to the carotenoid (siphonoxanthin) pigment absorption of green light along with the higher penetration ability of green wavelength in dense cultures as compared to blue and red lights (101).

In the present study, the supplemented green LED light improved the growth rate in comparison to the control group (no green light supplementation) but not to significant difference level and this means that the green light supplementation was not effective in improving the growth of *C. reinhardtii*. Two possible explanation can be conducted for our results:

1) The green light is known for its penetration deep into large and dense algae cultures in comparison to red and even blue light (102) and thus it results in higher growth rate as reported by de Mooij et al., (65). However, the small sample size used in this study (low density culture) and the constant mixing may have prevented the efficient effect of green LED light in penetrating deep into the culture and thus its effect was not significant in comparison to control group.

2) Under high light intensity (above 100 μmol/m^2.s), the blue and red lights may cause photoinhibition and possibly saturation effect which could be counteracted by green light supplementation (103) (104). the light intensity used in this experiment was low (40 μmol/m^2.s) and thus the photo-inhibition and saturation effects of blue or red lights that could have been compensated by green light were not observed in the cultures and for this reason, green LED light supplemented samples where not significantly higher than cultures grown in only red and blue light combination.

Other studies regarding the effect of green LED light on microalgae used different light intensities from this study, Ajayan et al., (105) states that high-intensity green light (above 70 μmol/m^2.s) expands the photosystem saturation and enhances the lipid content under high intensity stress conditions in *C. reinhadtii*. Furthermore, Jung et al. (85) used green LED light with 100 μmol/m^2.s intensity as a stress condition on three microalgae species (*Phaeodactylum tricornutum*, *Dunaliella tertiolecta*, and *Isochrysis galbana*), and about 50% (w/w) increase in lipid accumulation was found in all the cultures grown under green light. However, Vadiveloo et al., (64) used green wavelength in 30 μmol/m^2.s intensity on *Nannochloropsis sp.,* and no growth was observed.

Thus, the light intensity is a crucial aspect in examining the effect of certain wavelength on the growth of microalgae. Using low light intensity (below 50 μmol/m^2.s) may prevent detecting the positive effect of green light. On the other hand, using high light intensities (above 100 μmol/m^2.s) only displays the green light as a stress condition and its effect on lipid accumulation regardless its effect on photosynthesis process.

However, while there were no statistical differences in the growth of the green light supplemented cultures as compared to control group, none of the groups showed a lower growth than the control group containing only red and blue light despite having lower red and blue ratios that reached to 37% lower than control group. This suggests a certain compensation mechanism by the green light in case of low blue and red lights.

Certain accessory pigments have been reported to absorb green wavelength and transfer the energy to chlorophyll pigments for photosynthesis in case of low light intensity. These pigments include carotenoids like xanthophylls which absorbs blue to green light (106). The absorbance of green light by these pigments and participate in photosynthesis could explain the compensation of green light in the growth in case of low blue and red light. Nevertheless, more studies are required in the mechanism of harvesting green light spectrum in both algae and plants.

There is a lack of molecular level studies regarding the effect of green light supplementation on the photosystem in microalgae, the only study was by Kalmaoğlu (87) which included monitoring the effect of green light supplementation on certain genes related to photosynthesis along with cell count and chlorophyll level in *Haematococcus pluviali,* the results showed that 10% green light supplementation significantly increased all the growth parameters and the following genes: 1) RubisCo gene (responsible for carbon assimilation), 2) PsbS (controls cyclic electron flow to PSI and NPQ process). While 20% green light caused downregulation of these genes.

5.4 Chlorophyll and Green LED Light Supplementation

A study by Mohsenpour and Willoughby (102) showed that chlorophyll production was stimulated by green light in *C. vulgaris*, while it was not affected in *G. membrancacae*. in the present study, the results showed similarity between *C. vulgaris* species and *C. reinhardtii* as the green light produced a significantly higher chlorophyll levels in green light supplemented cultures in comparison to control group.

Chlorophyll pigments are known to have their peak absorption under blue and red wavelengths and reflects green light giving it its known color (31). So, under higher green light fractions and lower red/blue ratios it is expected to have lower

chlorophyll pigments. This was not the case in this study as the green-supplemented groups showed a significantly higher chlorophyll content as compared to control group (red and blue lights). Similarly, a study by Gaytán-Luna et al. (88) on *C. reinhardtii* showed that green light illumination resulted in three-fold increase in the chlorophyll content as compared to white light.

These results suggest a compensation mechanism by *C. reinhardtii* under different light illumination. The availability and intensity of light have a significant on the pigment content of microalgae, Chlorophyll content decreases with high light intensity during photo-adaptation process, and vice versa (107). Due to their high absorption capacity, blue and red lights result in a lower chlorophyll level. Conversely, cultures supplemented with green light are suggested have higher chlorophyll levels to absorb more of the available light and weekly absorbed green light. Moreover, the enhancement of green light to chlorophyll levels could be related to the previously discussed effect of xanthophyll pigments in absorbing the green wavelength under limited light and transfer it through xanthophyll cycle to chlorophyll pigments (figure 1.7) and consequently improving the photosynthesis process. However, there is a lack of studies regarding this subject.

6. CONCLUSIONS AND RECOMMENDATIONS

Researches on plants and algae using LED light had provided an advantage in improving the knowledge of the optimal wavelength for development and metabolite production. Furthermore, the published green light studies focused only on its effect on lipid production. The aim of this study was to optimize the red and blue light combinations to achieve the highest growth and chlorophyll content in *C. reinhardtii*. Furthermore, the effect of green light supplementation on the expense of red and blue lights was studied to investigate its effect on the growth and chlorophyll levels.

In the present study, the results showed that the combination of red and blue light significantly enhanced the growth and chlorophyll production in green microalgae *C. reinhardtii* in an additive-effect manner in comparison to monochromatic and white light.

Moreover, the study's findings suggest that green light participates in compensation mechanism in case of low red and blue light and improves the growth and chlorophyll production in *C. reinhardtii*. However, further studies are required on green light harvesting mechanism of by algae and plants and its effect in a molecular level on the photosystem.

Finally, Green light should be considered for its use in indoor cultivation of microalgae under LED lighting system.

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