BOLU ABANT IZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY

EFFECTS OF GREEN LIGHT SPECTRUM ON GROWTH AND GENE EXPRESSION PROFILES OF GREEN ALGAE *HAEMATOCOCCUS PLUVIALIS*

MASTER THESIS OF SCIENCE

GÜLAY KALMAOĞLU

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APPROVAL OF THE THESIS

EFFECTS OF GREEN LIGHT SPECTRUM ON GROWTH AND GENE EXPRESSION PROFILES OF GREEN ALGAE *HAEMATOCOCCUS* PLUVIALIS submitted by **GÜLAY KALMAOĞLU** and defended before the below named jury in partial fulfilment 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 07.08.2020** by

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DECLARATION

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Gülay KALMAOĞLU

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ABSTRACT

EFFECTS OF GREEN LIGHT SPECTRUM ON GROWTH AND GENE EXPRESSION PROFILES OF GREEN ALGAE *HAEMATOCOCCUS PLUVIALIS*

MSC THESIS
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BOLU, AUGUST 2020

This study was carried out to reveal both growth and genetic effects of different ratios of green light on *Haematococcus pluvialis* cultures. *H. pluvialis* cells were grown and examined on 2 different experimental setups. Although there were differences in green light ratios in both experimental setups, cultures were grown at a total light intensity of 20 µmol m^{-2} s^{-1}.

In the first experiment setup, White light (W) treatment was used as the control group. In another treatment (G/2), the green light ratio in the white light illumination has been reduced by 50%. In the last treatment (G0), the rate of green light in white light was reduced to 4%. G0 treatment has increased in terms of cell number, chl-a amount, biomass and dry weight compared to other treatments, and there is a statistically significant difference compared to other treatments.

In the second experiment setup, Red-Blue (RB) light mixture was used as the control group. In the other treatment has been used G10 as 10% green light supplementation to the RB light mixture. In the last treatment has been used G20 as 20% green light supplementation to the RB light mixture and the research was carried out. When the number of cells, chl-a amount, biomass and expressions of certain genes (PsaB, PsbS, $PTOX_2$ and Rubisco) were examined, G10 treatment was observed more clear and higher growth and more expression levels of genes (except $PTOX_2$ gene).

In the G10 treatment was observed significant differences than other treatments as statistically.

KEYWORDS: LED, Green Light, Gene Expression, *Haematococcus pluvialis*, qRT-PCR

ÖZET

YEŞİL IŞIK SPEKTRUMUNUN *HAEMATOCOCCUS PLUVIALIS* YEŞİL ALG TÜRÜNÜN BÜYÜMESİNE VE GEN İFADELERİNE ETKİSİ
YÜKSEK LISANS TEZI
GÜLAY KALMAOĞLU
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
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BOLU, AĞUSTOS - 2020

Bu çalışma, *Haematococcus pluvialis* kültürlerinde farklı yeşil ışık oranlarının hem büyüme hem de genetik etkilerini ortaya çıkarmak için yapıldı. *H. pluvialis* hücreleri büyütüldü ve 2 farklı deney düzeneğinde incelendi.Her iki deney düzeneğinde de yeşil ışık oranlarında farklılıklar olmasına rağmen, kültürler 20 µmol m^{-2} s^{-1} toplam ışık yoğunluğunda yetiştirildi.

İlk deney düzeneğinde kontrol grubu olarak Beyaz ışık (W) tedavisi kullanıldı. Başka bir tedavide (G/2), beyaz ışık aydınlatmasındaki yeşil ışık oranı% 50 azaltılmıştır. Son tedavide (G0), beyaz ışıkta yeşil ışık oranı% 4'e düşürüldü. G0 tedavisi hücre sayısı, chl-a miktarı, biyokütle ve kuru ağırlık açısından diğer tedavilere göre artmıştır ve diğer tedavilere göre istatistiksel olarak anlamlı bir fark vardır.

İkinci deney düzeneğinde kontrol grubu olarak Kırmızı-Mavi (RB) ışık karışımı kullanıldı. Bir diğer tedavide (G10), RB ışık karışımına % 10 yeşil ışık takviyesi olarak kullanılmıştır. Son tedavide ise (G20) RB ışık karışımına% 20 yeşil ışık takviyesi kullanılmıştır ve araştırma yapılmıştır. Hücre sayısı, chl-a miktarı, biyokütle ve belirli genlerin (PsaB, PsbS, $PTOX_2$ ve RubisCo) ekspresyonları incelendiğinde, G10 tedavisinin daha net ve daha yüksek büyüme ve daha fazla gen ekpresyon seviyeleri gözlemlenmiştir ($PTOX_2$ geni hariç).

G10 tedavisinde istatistiksel olarak diğer tedavilere göre önemli farklılıklar gözlemlenmiştir.

ANAHTAR KELİMELER: LED, Yeşil Işık, Gen İfadesi, *Haematococcus pluvialis, qRT-PCR*

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LIST OF ABBREVIATIONS AND SYMBOLS

ADP : Adenosine diphosphate

ANOVA : Analysis of variance

Anth : Anthaxanthin

ATP : Adenosine triphosphate

BG-11 : Blue-green (culture medium) No.11

c : Speed of light (299,792,458 m s^{-1})

Car : Carotenoids

CBC : Calvin-Benson cycle

cDNA : Complementary DNA

Chl : Chlorophyll

CO_2 : Carbondioxide

Cyt b_{559} : Cytochrome b559

$Cytb_6f$: Cytochrome b_6f complex

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

dNTPs : Deoxyribonucleoside triphosphate

DW : Dry Weight

EDTA : Ethylenediaminetetraacetic acid

Fe^{2+} : Iron(II)

FLs : Fluorescence lamps

G/2 : 50 % green light subtraction

G0 : 96 % green light subtraction

G10 : 10% green light supplementation

G20 : 20% green light supplementation

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

Glyceraldehyde-P : Phosphoglyceraldehyde

Glycerate-bis-P : Diphosphoglycerate

Gly-P : Phosphoglycerate

h : Max Planck constant (6.626 x 10-34J s^{-1})

H. pluvialis : *Haematococcus pluvialis*

H_2S	**:** Hydrogen sulfide
LED	**:** Led Emitting Diodes
mRNA	**:** Mesenger RNA
$NADPH_2$	**:** Nicotinamide ‑ adenine dinucleotide phosphate
nm	**:** Nanometer
NPQ	**:** Nonphotosynthetic quenching
OD	**:** Optical Density
P680	**:** Primary electron donor in PSII
P700	**:** Primary electron donor in PSI
PBS	**:** Phosphate Buffered Saline
PPFD	**:** Photosynthetic photon flux density
PQ	**:** Plastoquinone pool
PRC	**:** Photosystem reaction centre
PSI	**:** Photosystem I
PSII	**:** Photosystem II
$PTOX_2$	**:** Plastid terminal oxidase 2
Q_A and Q_B	**:** Primary and secondary quinone acceptors
qPCR	**:** Quantitative polymerase chain reaction
RB	**:** Red and Blue light
RGB	**:** Red-Green-Blue light
Ribulose-bis-P	**:** Ribulose bisphosphate
Ribulose-P	**:** Ribulose phosphate
RNA	**:** Ribonucleic acid
ROS	**:** Reactive oxygen species
rpm	**:** Revolutions per minute
Rubisco	**:** Ribulose biphosphate carboxylase/oxygenase
SD	**:** Standard Deviation
TBE	**:** Tris/Borate/EDTA
Triose-P	**:** 3-carbon products
TyrZ	**:** Tyrosine Z
UV	**:** Ultraviolet
W	**:** White Light
λ	**:** Wavelength of light (m)
®	**:** Register

LIST OF UNITS AND SYMBOLS

Table 1. List of basic parameters used.

Parameters	Name of units	Symbol of units
Mass	Gram	g
Molecular Unit	Base pair	bp
	Molar [mol L^{-1}]	M
	Mole	mol
Temperature	Degree Celsius	°C
Time	Day(s)	d
	Hour(s)	h
	Minute(s)	min
	Second(s)	s
Volume	Liter	L

Table 2. List of decimal sub-/ multiples of units.

Sub-/ Multiple of unit	Name of prefix	Symbol of prefix
Submultiple		
10^{-12}	Pico	p
10^{-9}	Nano	n
10^{-6}	Micro	µ
10^{-3}	Milli	m
10^{-2}	Centi	c
Multiple		
10^{3}	Kilo	k
10^{6}	Mega	M
10^{9}	Giga	G

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1. INTRODUCTION

1.1 Microalgae

Microalgae are the primary producer of the aquatic food chain and highly diverse polyphyletic groups of single-cell photosynthetic organisms. They can grow in a range of aquatic habitats, including lakes, ponds, rivers, oceans, and even wastewater. They contribute almost 50% of atmospheric oxygen (Harlin and Darley, 1988). They are well-adapted organisms and survive under a large spectrum of environmental stresses—including heat, cold, drought, salinity, photo-oxidation, anaerobiosis, osmotic pressure and UV exposure (Tandeau-de-Marsac and Houmard, 1993). Algae are classified according to morphological characters as microalgae and macroalgae. Microalgae are prokaryotic or eukaryotic single-cell forms and generally classified in 4 groups; *Rhodophyta* (red algae), *Chrysophyceae* (golden algae), *Phaeophyceae* (brown algae), and *Chlorophyta* (green algae). Macroalgae (seaweed) are multicellular, large-size eukaryotic forms of organisms. Although the majority of algae species are photosynthetic life forms, heterotrophic, mixotrophic, symbiotic and even parasitic life forms are recorded in the literature (Lee et al., 2008).

Algae have received great interest in the field of biotechnology because of their ability to synthesis economically valuable bioactive molecules as primary and secondary metabolites such as carotenoids, omega-3, and phycobiliproteins since the 1950s (Borowitzka and Borowitzka, 1987; Burlew, 1953). For example, species of *Arthrospira genus* (called as *Spirulina*) has been used in both human and animal nutrition because of high protein and carotene content of the cell (Soletto et al., 2005). *Chlorella* species is known for its free radical scavenging and lowering blood lipids due to its β-1,3-glucan content. It is also known for the healing of ulcers and wounds, high blood pressure and antitumor properties (Iwamoto, 2004; Gouveia et al., 1996; Yamaguchi, 1997). *Dunaliella salina* has been used in human consumption due to its β-carotene content (Metting, 1996). *Arthrospira* and *Porphyridium* are known to be the main source of phycobiliproteins. It is preferred in the

pharmaceutical, cosmetic and colouring sectors due to the phycocyanin and phycoerythrin pigments they contain (Viskari and Colyer, 2003).

Carotenoids are one of the most important biotechnological pigments and important in intracellular protection for microalgae. According to the previous studies, carotenoids received great attention with their antioxidant properties in the immune system, taking precautions against various cancers as well as supporting as vitamin A (Bendich, 1989; Peto et al., 1981; Van Poppel, 1993). There are more than 600 carotenoids and still, new carotenoids are added to this list (Ong and Tee, 1992; Mercadante, 1999). Carotenoids are divided into two as carotene and xanthophyll. β-carotene is given as an example of carotene while xanthophylls are zeaxanthin, lutein, canthaxanthin and astaxanthin. It is known that these various carotenoids are produced by microalgae such as *Dunaliella salina*, Nannochloropsis, *Haematococcus pluvialis*, *Chlorella zofingiensis* (Bhosale and Bernstein, 2005).

1.1.1 *Haematococcus pluvialis* Flotow

H. pluvialis were first carried by Girod-Chantrans in 1797 and identified as a new species by Flotow in 1844 and redefined by Braun in 1851 with corrections (Lorenz, 1999). Systematic classification of *H. pluvialis* is given below.

Phylum: Chlorophyta
Class: Chlorophyceae
Order: Volvocales
Family: Haematococcaceae
Genus: Haematococcus
Species: *Haematococcus pluvialis* Flotow, 1844

1.1.1.1 Biology and Life Cycle of *H. pluvialis*

H. pluvialis is a eukaryotic, unicellular, motile, biflagellate green freshwater algae. They are able to live at both phototrophic and autotrophic conditions. *H. pluvialis* have four cellular morphology (macrozooids, microzooids, palmella, and

hematocysts or called as aplanospores) in two different life stages in green vegetative motile and red non-motile encysted stages when environmental conditions change (Elliot, 1934) (Figure 1.1). Under favourable conditions, cells predominantly are fast-growing motile macrozooids forms with two flagella in the green vegetative growth stage. Under unfavourable conditions induced by stress factors such as nutrient limiting, excess lighting, salinity, oxidative stress, temperature, macrozooids lose their flagella and increase their cell volume accompany with the termination of cell division and developing of the resistant thick cell wall (Kobayashi et al., 1997a; Boussiba, 2000). Cells are formed into non-motile palmella and enter the vegetative resting stage. If these stress conditions are persisted, palmella is transformed into asexual non-motile aplanospores forms and accumulating high amount of astaxanthin in lipid droplet in the cytoplasm that is known as red non-motile cyst phase.

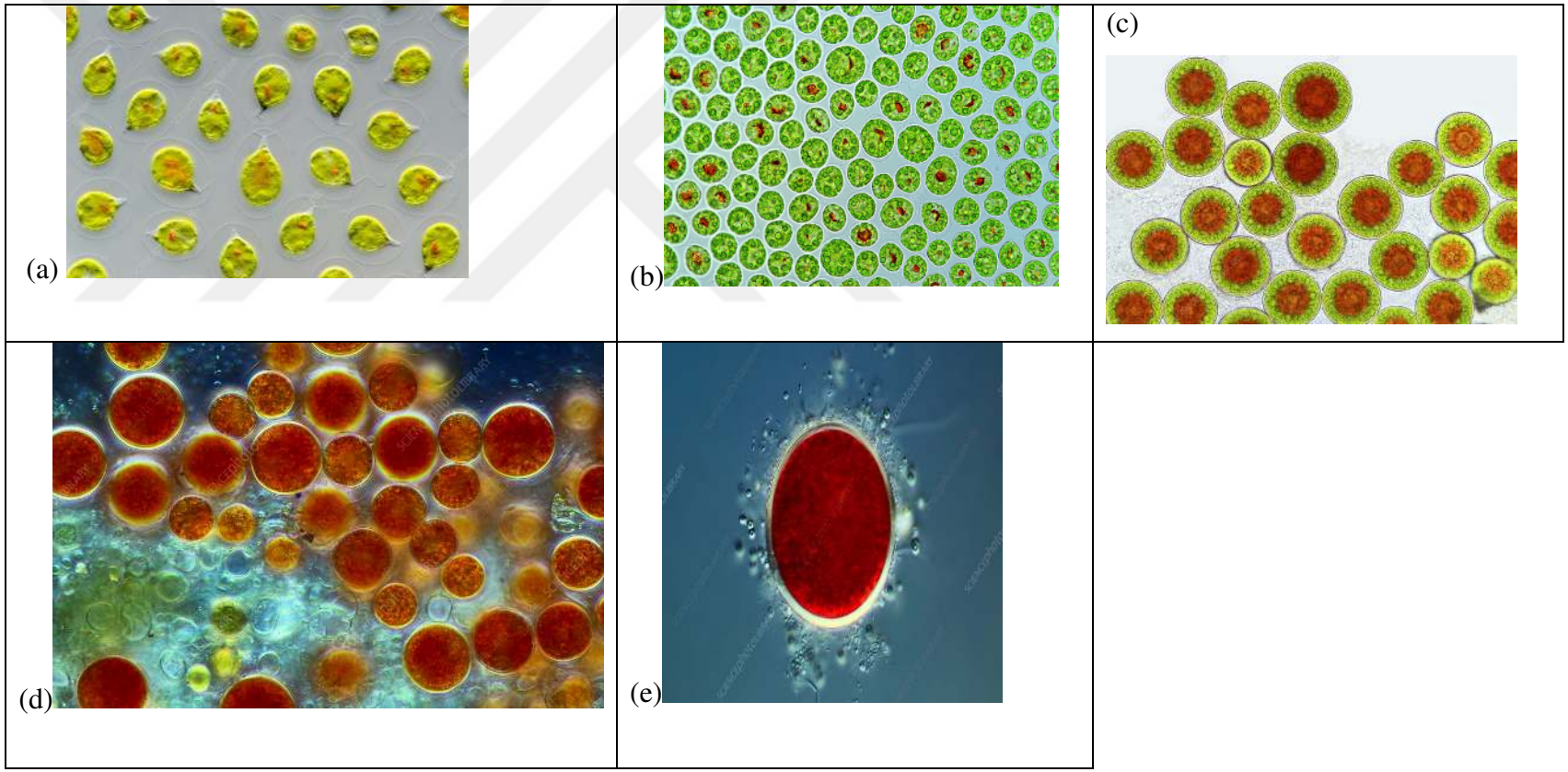


Figure 1.1. Life stages of *Haematococcus pluvialis* a) macrozooid phase, b) microzooid phase, c) Palmella phase and d) and e) aplanospore phase (accessed from (Micro-foto, 2019) (http://www.mikro-foto.de/).

Red aplanospores can be turned into flagellated macrozooids or produce microzooids (Figure 1.2) after gametogenesis if environmental conditions return to favourable (Boussiba, 2000).

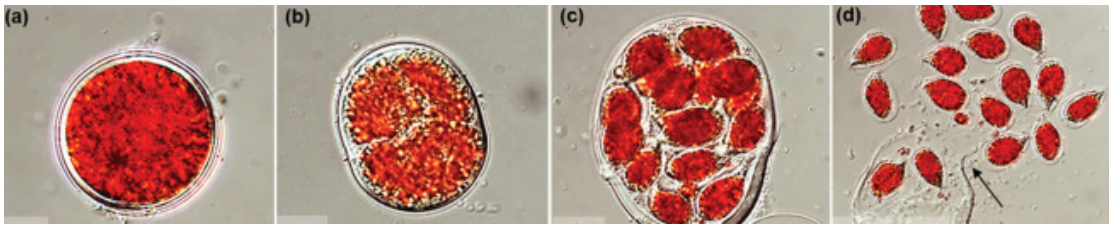


Figure 1.2. Germination of *H. pluvialis* cysts a) Mature cyst, b) Dividing cyst with cell divisions c) Dividing cyst with differentiated zooids, d) Release of zooids from the cyst (Praveenkumar et al., 2015).

1.1.2 Economically Importance of *Haematococcus pluvialis*

H. pluvialis is known as the organism of the highest capability of natural astaxanthin biosynthesis (up to 4% of dry weight) for industrial applications (Li et al., 2011). Astaxanthin ((3S-3'S)-dihydroxy-β,β,-carotene- 4,4'-dione) is a member of xanthophyll family of a carotenoid and also naturally synthesized in some plant, algae, and bacteria (Boussiba and Vonshak, 1991; Johnson and An, 1991). It has economic importance in the nutraceutical, pharmaceutical, and food industries due to high antioxidant, anti-inflammatory and antitumor properties (Higuera-Ciapara et al., 2006). Astaxanthin is used for pigmentation of many commercially reared aquatic animals such as salmonids, shrimp, lobster and crayfish that cannot synthesize de novo (Lorenz and Cysewski, 2000). Furthermore, it has been considered potential medicine due to its high-level antioxidant capacity (Kobayashi et al., 1997a; Hussein et al., 2006).

1.1.3 Biotechnology Applications in *Haematococcus pluvialis*

In commercial applications, the two-stage of culture system of *H. pluvialis* has commonly been used for the production of natural astaxanthin from. In the green stage, it is aimed to have a high biomass of green cell in optimum growing

conditions. Then hyperaccumulation of astaxanthin production in cyst form is accomplished by application of stress factors such as salt addition and high light intensity or nutrient deficiency (Sarada et al., 2002; Li et al., 2010; Xi et al., 2016). Thus, growth conditions of *H pluvialis* in the green stage is a critical factor in astaxanthin production. Factors affecting on photosynthesis rate and carbon assimilation of *H. pluvialis* have great attention in algae biotechnology and algae research (Nagaraj et al., 2012; Kobayashi et al., 1992a).

1.2 Photosynthesis in Microalgae

Photosynthesis basically is a process of converting sunlight energy to extract proton and electron from different molecules such as Fe^{2+}, H_2S and H_2O to reduce CO_2 to form organic molecules (Masojidek et al., 2013). The photosynthesis process is a photo-chemical situation with anoxygenic (photosynthetic bacteria) and oxygenic organisms (algae, cyanobacteria, and plants) turn to light energy into chemical energy. So, light is the basic source of energy for photosynthetic organisms. Algae are the first oxygen-producing photosynthetic microorganisms evolved 2 billion years ago and shape oxygenous atmosphere in the Earth. Photosynthetic algae are classified into four groups *Rhodophyta* (red algae), *Chrysophyceae* (golden algae), *Phaeophyceae* (brown algae), and *Chlorophyta* (green algae) according to pigments used for light harvesting (Staehelin, 1986). Absorption of light energy in a photosynthetic organism is based on existed pigments in the organism. There are three major light energy harvesting pigments chlorophyll (chl), carotenoids and phycobilins localized into the chloroplast organelle containing lipoprotein membrane (thylakoids) and stroma (Staehelin, 1986). Chlorophylls are the main pigment for light-harvesting in the photosynthesis process (Hoek et al., 1995). There are different types of chl (chl-a, chl-b and chl-c), but chl-a is the dominant pigment in green algae species. Chlorophyll pigments mainly absorb blue or blue-green colors of 450-475 nm and red colors of 630-675 nm of visible light spectrums (Figure 1.3) appeared in the characteristic of green color. Other pigments which are carotenes, phycobilins and xanthophylls are known as accessory pigments and they provide absorption of photons and transfer of energy to chlorophylls (Purves and Orians , 1983).

Carotenoids pigments have an absorption range between 400 and 550 nm of the light spectrum appear in yellow-orange (Figure 1.4).

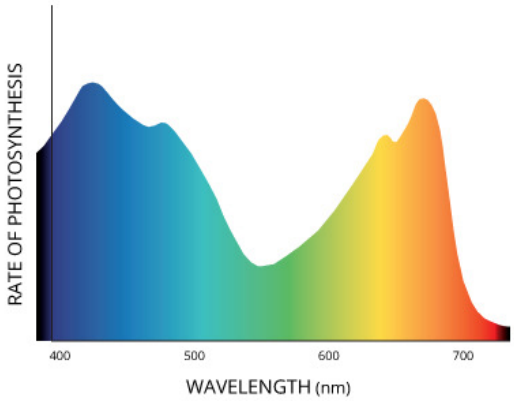


Figure 1.3. Visible photosynthetic light spectrum.

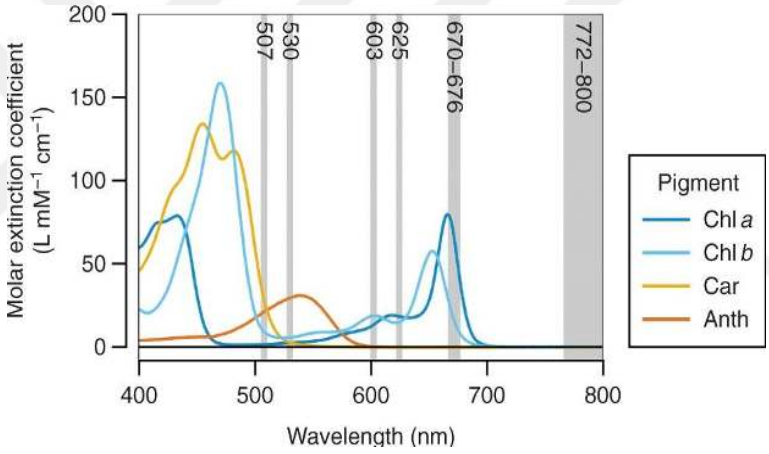


Figure 1.4. Absorption spectrum of different pigments (Lichtenthaler, 1987).

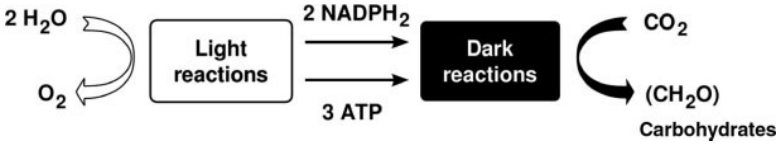


Figure 1.5. The light and dark reactions of photosynthesis. (Masojidek et al., 2013).

Photosynthesis is classically divided into 2 processes; light and dark reactions (Figure 1.5). The light reaction is a process of converting light energy into chemical energy as a biochemical reductant of $NADPH_2$ and energy source of ATP; the dark reaction is a process of utilizing $NADPH_2$ and ATP in the biochemical reduction of CO_2 for carbon assimilation.

1.2.1 The Light Reaction of Photosynthesis

Primary light reactions of photosynthesis occur in the thylakoids which is a complex membrane system embedded in the chloroplast. Primary photosynthetic reactions of photosynthetic electron transport and photophosphorylation are mainly regulated by five major proteins and pigment systems which are light-harvesting antenna, photosystem II (PSII), the cytochrome b_6f complex ($Cytb_6f$), photosystem I (PSI) and ATP synthase embedded in the thylakoid membrane (Figure 1.6). In green algae PSII and PSI have their own light-harvesting complex systems binding Chl-a and Chl-b called LHCII and LHCI respectively. Outer light excitation energy is collected by both LHCII and LHCI systems to transfer to the photosystem reaction centre (PRC) (Masojidek et al., 2013).

The light energy is captured by PSI and PSII that operate in series of connected by a chain of electron carriers having redox potential proceeded energetically downhill from a lower to a higher potential. Upon exposure of light energy, photophosphorylation is proceeded by simultaneous electrons and proton charge transfer across the thylakoid membrane. Two electrons extracted from H_2O are transferred through the chain of electron carrier to the plastoquinone pool (PQ), $Cytb_6f$ and PSI, while protons are transported across the thylakoid membrane from stroma into the lumen (inside of thylakoid) to produce $NADPH_2$ and ATP respectively to be used in Calvin-Benson cycle (CBC) for CO_2 assimilation (Masojidek et al., 2013).

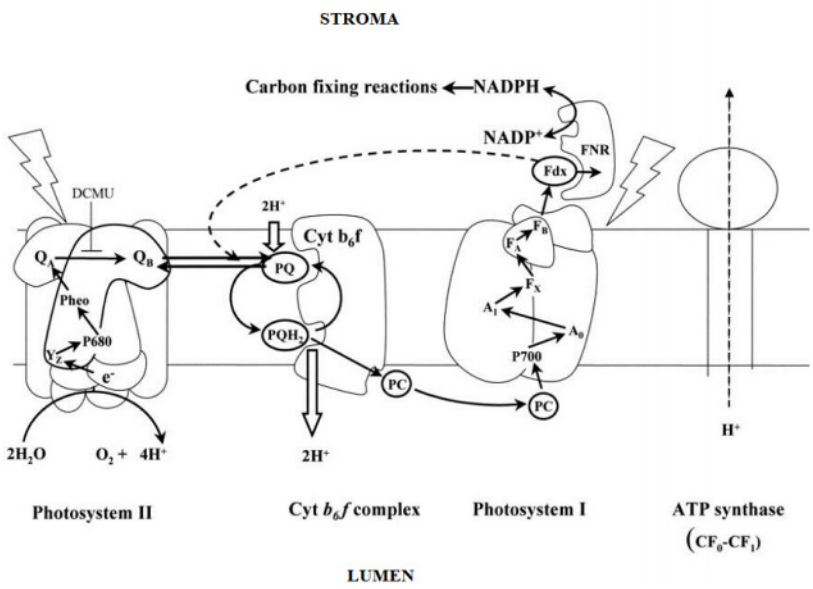


Figure 1.6. This schema is shown that electron transferring in the photosynthesis process (Rob, 2014).

PSII is located in the thylakoid membrane and consist of the reaction center, the oxygen-evolving center and inner light-harvesting antenna. Reaction center of PSII contains D1 and D2 proteins and α and β subunits of Cyt b_{559}, tyrosine Z, P680, Q_A and Q_B (pheophytin and the primary and secondary quinone acceptors, respectively).

Electron transport system between PSII and PSI occurs through $Cytb_6f$ complex in two steps, in the first step; two electrons provided by Plastoquinones (PQ) for the electrons transfer between PSII and $Cytb_6f$, simultaneously two protons are translocated from the stroma into the lumen by PQ. In the second step electron transfers from $Cytb_6f$ to PSI is performed by Plastocyanin in the thylakoid lumen.

PSI is an intermembrane complex operating reducing redox potential to reduce ferredoxin to produce $NADPH_2$. Two proteins PsaA and PsaB are located in the reaction center and acting as prosthetic cofactors in the electron transfer system. Primary electron charge separation is initiated in a Chl dimer P700 in PSI complex. Chl-a, phylloquinone and F_x (4Fe-4S) serve as an electron carrier in the PSI system. Electron is transferred through 4Fe-4S, F_A, F_B, and ferredoxin as the final electron acceptor.

ATP synthase is composed of two subunits of CF_0 and CF_1 served as a membrane enzyme to catalyzes ATP synthesis. CF_0 perform as a proton channel to transfer through CF_1 which catalyzes ATP synthesis.

1.2.2 The Dark Reaction of Photosynthesis

1.2.2.1 Carbon Assimilation

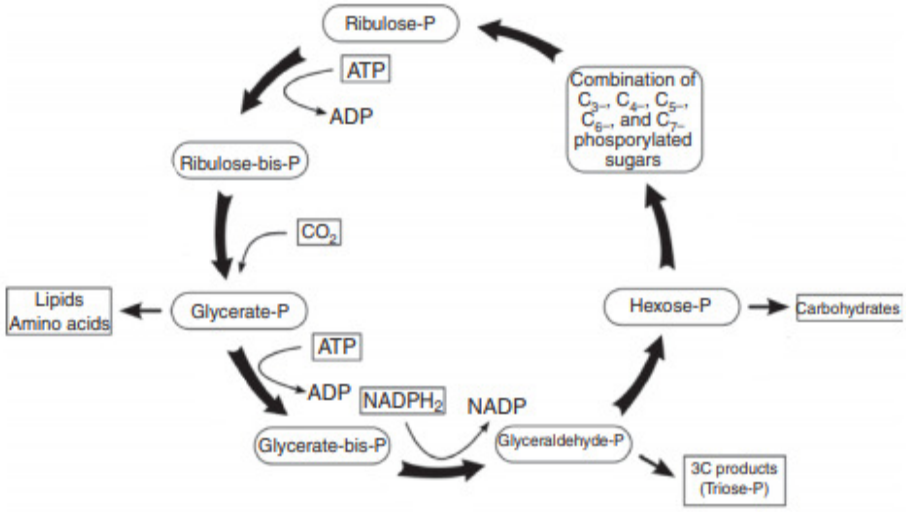


Figure 1.7. The photosynthetic carbon fixation pathways - The Calvin-Benson cycle (Masojidek et al., 2013).

$NADPH_2$ and ATP produced in light reactions are used for fixation of CO_2 in the dark reaction by Calvin and Benson biosynthesis cycle. (Figure 1.7). The Calvin and Benson Cycle reactions are initiated by adding CO_2 to ribulose biphosphate to form phosphoglycerate (Gly-P). The reaction is catalyzed by ribulose biphosphate carboxylase/oxygenase (RubisCo).

There are four phases for occurring the level of sugar from the CO_2 fixation in the Calvin-Benson cycle as Carboxylation phase, Reduction phase, Regeneration phase and Production phase. In the carboxylation phase; ribulose bisphosphate (Ribulose-bis-P) which contain 5-carbon sugar with CO_2 consists of two molecules of phosphoglycerate (Glycerate-P) whereby the enzyme ribulose bisphosphate carboxylase/oxygenase (called RubisCo). In the reduction phase separate two steps

for forming 3-carbon products (Triose-P) from phosphoglycerate. After diphosphoglycerate and ADP from via phosphorylation of phosphoglycerate with adding ATP, Phosphoglyceraldehyde (Glyceraldehyde-P) with adding to $NADPH_2$ from the reduction of diphosphoglycerate (Glycerate-bis-P). In the regeneration phase, ribulose phosphate (Ribulose-P) is occurred with combining 3-, 4-, 5-, 6-, and 7-carbon sugar phosphates by transketolase and aldolase enzymes. In the production phase; carbohydrates, fatty acids, amino acids, and organic acids produced in photosynthetic CO_2 fixation (Masojidek et al., 2013).

1.3 Light-harvesting Pigments in Algae

Almost all algae are photosynthetic and comprise both light and dark reactions (Calvin cycle). In dark reaction, carbon dioxide (CO_2) is bound to ribulose biphosphate by ribulose biphosphate carboxylase enzyme as an initial step of glycose biosynthesis like in terrestrial plant species. However light reactions show some differences in algae species from terrestrial plant species due to different light-harvesting systems processed by different pigments in algae (Hiller et al., 1991). Blue and red light are absorbed mostly by chlorophyll and less by phycobiliproteins pigment, carotenoids pigments absorb mainly blue and green light. Therefore, the rate of light-harvesting and photosynthesis for algae species depends on pigment composition, pigment concentration and availability of light wavelength in their surroundings (Kelling, 2013). Chlorophyll-a has commonly used pigments by almost all algae species to collect photosynthetically active light spectrum, additionally, chlorophyll-b is also used by green algae and euglenophytes for the photosynthesis. Different algae species can collect different light wavelength spectrum which cannot be absorbed by chlorophyll pigments using different pigments such as chlorophyllides, carotenoids and phycobiliproteins. For example, chromophyte algae, dinoflagellates, cryptomonads and species from Micromonadophyceae groups can use chlorophyllides pigments. Carotenoids are used by green algae and phycobiliproteins is used by red algae to collect photosynthetically active light spectrum (Marriott and Blankenship, 2011).

1.4 Light Distribution in Aquatic Systems

In the aquatic system, sunlight wavelengths show different penetration characteristics according to the depth. Red wavelengths are mostly absorbed within the first few meters of surface water in aquatic systems. Blue light absorption is depending on water transparency, in turbid water containing a high amount of organic madder is more rapidly absorbed in few meters than red light. Greenlight can penetrate the deeper part of the water column and it is a dominant light spectrum for deep waters (Figure 1.8).

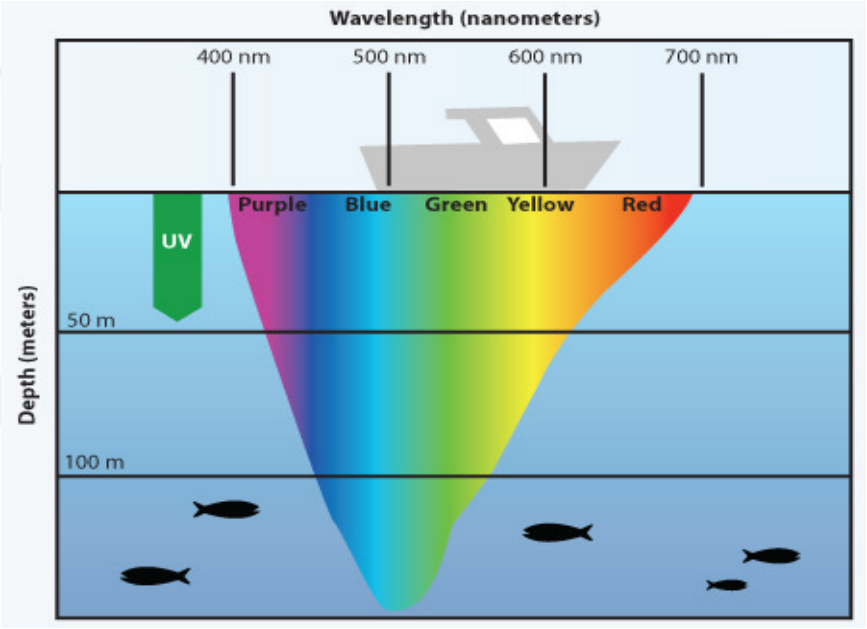


Figure 1.8. Light penetration in the aquatic system (Minnesota Sea Grant, 2019).

1.5 LED Light

In algae biotechnology, light requirements for algae growth in the mass cultivation of algae is generally provided by sunlight. Although sunlight energy is a free source for algae growth, it has several disadvantages in commercial applications. For example, sunlight is only present in sufficient intensities for photosynthesis throughout certain times of the year. Much of the energy is not able to use in the right wavelength both for photosynthesis and cannot be used to produce various metabolites. *H. pluvialis* requires light to grow and form astaxanthin and they can be

formed by artificial light or sunlight. Sunlight is cheaper than artificial light, but its energy intensity is restricted, and it can change to depend on diurnally and seasonally.

Artificial light, on the other hand, can provide the opportunity to work on the regulation of photosynthetic photon flux density (PPFD), the lighting time and the specific light spectrum. It is preferred to increase the quality of products and production in agriculture and industrial area. Usually, light-emitting diodes (LED) (Figure 1.9) and fluorescence lamps (FLs) (Figure 1.10) lighting systems are used as artificial light in microalgae researches.

FLs have wide emission spectrum and consisting of wavelengths with low photosynthetic activity in some of the microalgae (Carvalho et al., 2011).

LEDs feature monochromatic illumination that can be used in various and specific wavelengths. Therefore, it is easier to monitor the physiological and biochemical changes that may occur in species in a single wavelength or combination of wavelength in microalgae cultivation (Olle and Virsile, 2013). LEDs are not able to produce light in a broad white light spectrum which may make it necessary to use a combination of light sources or combination of LEDs.

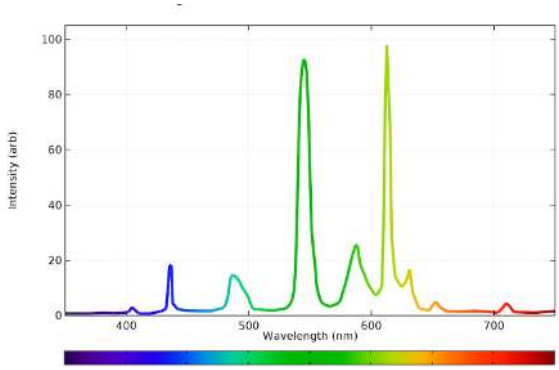


Figure 1.9. The emission spectrum of a typical LED

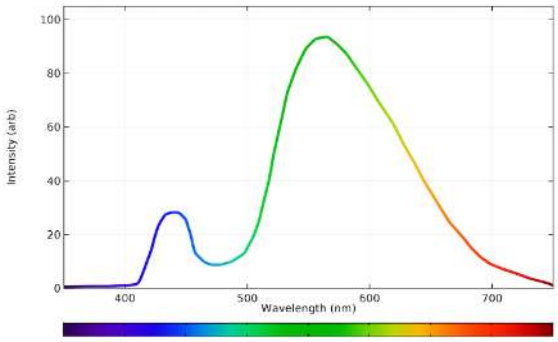


Figure 1.10. The emission spectrum of a typical fluorescent bulb (COMSOL, 2019)

There are several reasons why LEDs lights are preferred in algae cultivation;

1. Narrow spectral output
2. Light from high electric efficiency, which occur less heat
3. Small length and weight, which external or internal lighting for photobioreactor
4. It can be powered by low voltage for the safe condition
5. Long life expectancy
6. Asolid-state (Park and Lee, 2000).

Because of these advantages, LEDs are preferred for studying in single wavelengths such as red (625nm), green (525nm), blue (470nm) or combination of these wavelengths which is blue-purple(410nm) and purple (380nm) (Katsuda et al., 2004).

1.5.1 LED Lighting Mechanism

LEDs have two portions as a positive (p-type) layer and negative (n-type) layer (Figure 1.11). While p-layer has existed of acceptor dopant atom and so, it has a high amount of electron holes in the valance band, n layer has the presence of donor dopant atoms thus, it has excess electrons in the conduction band.

The operating mechanism of LEDs, the electrons in the n region cross the np junction region and join the hole in the p region. The heat and light energy produced

by the electrons as a result of this process is emitted from this junction region in the form of photons.

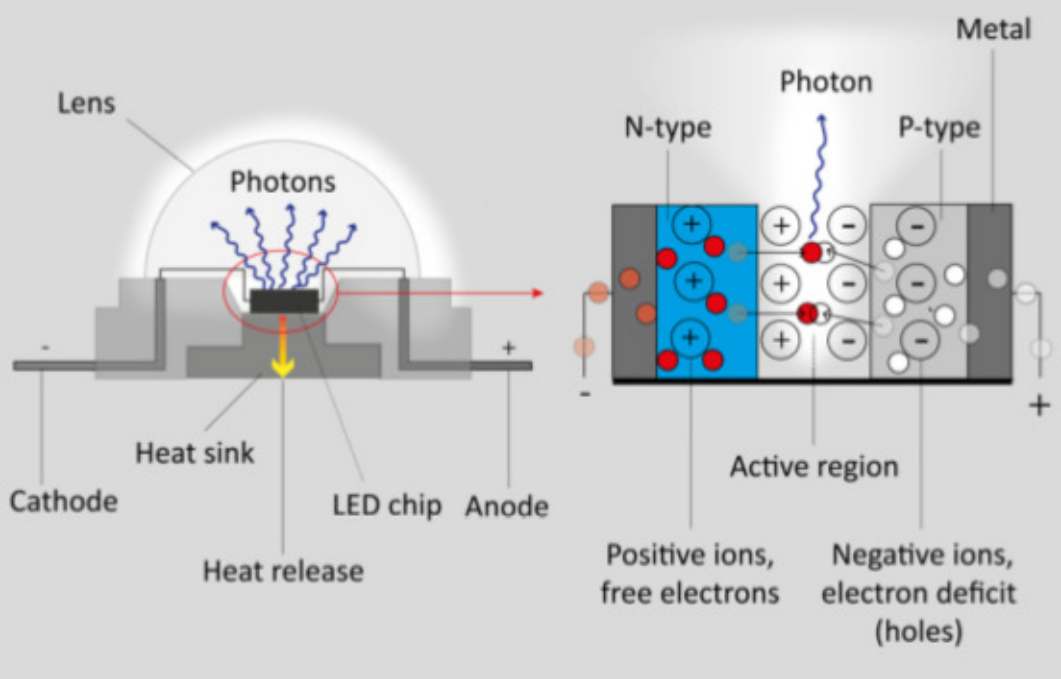


Figure 1.11. The operating mechanism of LEDs (Shulze et al.,2014).

Each photon has a certain energy, which can be defined as a function of its wavelength (Koc et al., 2013) (eq1).

$$E_\lambda = \frac{h \times c}{\lambda}$$

where = energy of a photon (J), h = Max Planck constant (6.626 x 10-34J s-1), c = speed of light (299,792,458 m s-1) and λ = wavelength of light (m).

Algae and plant are able to utilize with the wavelength between 380-750 nm for photosynthesis as efficiently. If energy from photons has more than 750nm, it is not efficiency to induce chemical reactions. If energy from photons has less than 380nm, it might ionization (Kommareddy and Anderson, 2003).

Red light can be captured by phytochromes which is a member of photoreceptors. Red light can increase of growth rate in microalgae and also land plant and promotes biomass production due to regulating of light-harvesting

mechanism for microalgae as well as flowering for plants (Kianianmomeni and Hallmann, 2014). Red light is essential for the growth of land plants, algae. But, cyanobacteria is able to utilize blue light less efficiently than algae and land plants due to having the loss of chlorophyll b by many cyanobacterial species (Tomitani et al., 1999). Phytochromes which is photoreceptors are detected by red to far-red light in algae and land plants.

Photons of blue light higher than required for using photosynthesis and are detected by cryptochromes. Hence, this light may occur stress for microalgae such as nonphotosynthetic quenching (NPQ) and reactive oxygen species (ROS). To protect these stress conditions, algae and plants produce photoprotective pigments such as xanthophylls, astaxanthin (Jahns and Holzwarth, 2012).

The green light is important for encouraging the growth of plant and microalgae. Red and blue light cannot be captured by all cells as shading occurs in cultures that have reached a certain cell density. But green light transition through culture more efficiently than blue or red light and supplementation of green light into red and blue light may contribute to the growing of microalgae. Greenlight which has long light path is less preferred by algae. When cultures reached highly density, the green light is able to travel deeper into the culture (Stadnichuk et al., 2011). The green light is not captured by photoreceptors such as red and blue light. However, the green light may be captured by phytochromes in the absence of lighting conditions (Chris, 2016).

According to recent research, it is observed that each light spectrum has different effects on microalgae. For example; in Katsuda et al., 2004 were shown that red LEDs are suitable for growing *H.pluvialis* cells and can be the induction of morphological changes of *H. pluvialis* cells.

1.5.2 Green Light Supplementation

Unlike blue and red light, green light passes through plant tissue more efficiently. Likewise, in aquatic ecosystems where have high algae growth, blue and

red light is absorbed within a few cm, but the green light is able to penetrate the deeper part of the water column. Therefore, it has been speculated that green light may partly promote primary production in the deeper part of aquatic systems where blue and red-light spectrum is not present. However, several studies reported suppression of green light on plant species especially when it is used as a monochromatic spectrum (Vadiveloo et al., 2015; Blair et al., 2014; Kendirlioglu and Cetin, 2017). For example, Went (1957) and Klein et al. (1965) tomatoes were grown in white and blue-red illuminated environments. According to the results, it was observed that the biomass of tomatoes grown in white light was less than that of tomatoes grown in the blue-red environment. The reason for this was reported to be due to the ratio of green light in the white light mixture. In the literature, there is only one study addressing a possible effect that enhanced plant growth if green light used a specific percentage together with red and blue light sources comparatively than white light sources (Kim et al., 2004). However, there is no more comprehensive research referring to such mechanisms of dose-dependent of green light effects on plant or algae species in the literature.

1.6 Information about of Candidate Genes (RubisCo, $PTOX_2$, PsaB, PsbS)

In this thesis, 4 gene expressions were investigated. The reference gene in this study was chosen as the 18s rRNA because of its stable expression profile in algae species in different environmental conditions (Hayashi, 2014). These 4 indicator genes were carried out to test how they affect the photosynthesis system, carbon assimilation and carotenoid biosynthesis due to changes in the amount of green light.

It was aimed to reveal the effects on the RubisCo gene which affects carbon assimilation, the PsaB gene to reveal the effect in the PSI system, PsbS gene which has a role in the PSII system and $PTOX_2$ gene which has a role in carotenoid biosynthesis.

RubisCo is found in organisms capable of photosynthesis, such as bacteria, archaea and eukaryotic algae. It is an enzyme responsible for the carbon assimilation

of CO_2 which is the most abundant source of carbon in nature by Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway (Tabita, 1999).

In studies conducted on species capable of photosynthesis, $PTOX_2$ has been reported to have two basic functions. The primary function, it has been reported to function in carotenoid synthesis. Another function is; It is to provide photochemical protection by triggering ATP production by providing electron flow through the PSII system, enabling the formation of NPQ (McDonald et al., 2011).

While the PsbS gene is found in plants and green algae, it is not found in photosynthetic organisms other than these groups (Büchel, 2015). It is known that the PsbS gene has a small effect on the NPQ system (Bonente et al., 2008b). PsbS gene product (PSII-S) is predicted to act as a chlorophyll-binding buffer system (Funk et al., 1995). Therefore, the expressions of the PsbS gene in plants grown in the dark have been investigated. It is known that the PSII-S protein binds to chl-a, chl-b and several carotenoids (Funk et al.,1994).

One of PSI's main centre centres is PsaB (Göhre et al., 2006). Studies have been carried out on the transcription of the PsaB gene to be informed about the molecular mechanism of chloroplast gene regulation that is light-dependent (Tullberg et al., 2000).

2. AIM AND SCOPE OF THE STUDY

This study aims to investigate the effects of green light spectrum on the growth of green algae *H. pluvialis*. The major aims are since green light is a spectrum that is rarely used by photosynthetic creatures, it has been tested to have information about whether changes in the amount of this light will increase the efficiency of the combination of red-blue light. At the same time, it has been tested to have information about whether green light can be effective in the development of cultures thanks to its high penetration.

Our hypothesis is based on a specific ratio of green light combined with red and blue light spectrums will enhance the growth rate of *H. pluvialis*. Green light is composed of the biggest portion in white light sources although it is not effectively used for photosynthesis. Green light proportion may be a determinant factor affecting the efficiency of light-harvesting rates of blue and red light in algae species. There would be dose-dependent mechanisms depending on green light ration in the photosynthetic process.

In order to test our hypothesis, we carried out two experiments by using RGB LED light sources controlled by Arduino codes to apply different green light portions on *H. pluvialis* liquid cultures. In the first experiments, green light ratios were subtracted by 50 and 100% in three different treatments to test if there is a suppressive effect of green light on the growth of *H. pluvialis*. In the second experiment, 10 and 20 % of green light supplementation were applied in red and blue light combination in order to test if there is a dose-dependent mechanism of green light on the growth of *H. pluvialis*.

Moreover, we carried out transcription expression profiles of 4 candidate genes PsaB, PsbS, RubisCo and $PTOX_2$, having central roles in PSI, PSII, carbon assimilation and carotenoid biosynthesis respectively to reveal putative molecular mechanisms altered by green light portions in red and blue light combinations.

3. MATERIALS AND METHODS

3.1 Cultivation of Microalgal Strain

3.1.1 Microalgal Strain And Culture Medium

H. pluvialis was obtained from the culture collection of algae at Gottingen University, Germany (SAG 34-1b). The stock culture was maintained in 250 mL Erlenmeyer flask containing 200 mL of BG-11 (Table 3.1) medium at the illumination of 20 µmol photon m^{-2} s^{-1} of white LED light of 16 h light - 8 h dark cycle. Cultures were continuously aerated by bubbling air with a mixture of 2 % CO2 under constant temperature at 25 ± 0.5 °C.

Table 3.1. Chemical Composition of BG-11 medium.

Component	Final Concentration
$NaNO_3$ (Merck 1.06535)	17.6 mM
K_2HPO_4 X $3H_2O$ (Merck 1.05099)	0.23 mM
$MgSO_4$ X $7H_2O$ (Merck 1.05886)	0.3 mM
CaCl2 X $2H_2O$ (Merck 1.02382)	0.24 mM
Citric acid ($C_6H_8O_7$) (sigma 251275)	0.031mM
Ferric Ammonium Citrate (sigma F-5879)	0.021 mM
$Na_2EDTA{\cdot}2H$ O (sigma E5184)	0.0027 mM
Na_2CO_3 (Merck 1.06392)	0.19 mM
Trace Metals Solution	
Thiamine- HCl (Vitamine B1) $C_{12}H_{17}Cl_2N_4OS$	0.30 µM

Components of BG-11 medium was prepared with distilled water by continuous stirring and pH level were adjusted to 7.2 – 7.4. Before adding thiamine-HCl vitamin mediums were autoclaved. For the long-term storage, algal cultures were maintained in a 1,5% agar medium without thiamine-HCl adding (Figure 3.1).

(a)

(b)

Figure 3.1. a)Liquid stock culture of *H.pluvialis,* b) solid stock culture of *H. pluvialis.*

3.1.2 Experimental Conditions And Led Light System

Two different experiments were conducted to investigate the effects of different green LED light spectrum compositions on the growth of *H. pluvialis*. Both experiments were conducted in 250 mL flasks in BG-11 medium with the same conditions of stock culture except for LED light combination. The experimental flask was shaken manually five times to prevent settlement. Light sources were located at the bottom of the culture flasks 2 cm away from the bottom of the flasks. Each

treatment in the experiments was contained with triplicated one control group and 2 experimental test group. Illumination of each RGB (Red Green Blue) LED light spectrum and their combination of percentage in total LED light were measured in dark room using OHSP-350 portable LED spectrometer (HOPOOCOLOR, China). Samsung 50/50 RGB led strip were used for LED illuminations in the experiments. The LED light was controlled by adjusting electric voltage connected to an Arduino based computer card and software programming.

3.1.2.1 Green Light Subtraction Experiment

Control groups (W) were illuminated with 20 µmol m^{-2} s^{-1} white light intensity containing red, green and blue LED light intensities of 8.31; 6,75; 5,39 20 µmol m^{-2} s^{-1} respectively. The ratio of RGB light intensity for white light was 31.3/61.1/4.6 respectively.

In the experimental groups, green light intensity was decreased as a fraction of blue light. The first experimental groups(G/2) were illuminated with 20 µmol m^{-2} s^{-1} in total containing red, green, blue LED light intensities of 11.61; 2.52; 6.82 µmol m^{-2} s^{-1} respectively. The ratio of Red and Blue light was kept as in white light condition and green light intensity were decreased to 32.5 % comparative to white light. The ratio of RGB light intensity for the first experimental groups was 59.1/32.5/8.4 respectively.

The second experimental groups(G0) were illuminated with 20 µmol m^{-2} s^{-1} in total containing red, green, blue LED light intensity of 12.80; 0.2; 7.91 µmol m^{-2} s^{-1} respectively. The ratio of Red, Green and Blue light was kept as in white light condition and green light intensity were decreased to 4.1 % comparative to white light. The ratio of RGB light intensities for the first experimental groups was 83.3/4.1/12.6 respectively. The red and Blue light ratio was 1.6 ± 0.1 for all treatments. The interface of chromatic diagram of x and y coordinate values for the LED light combination for all treatment in the first experiment were given in Figure 3.2 (a,b,c).

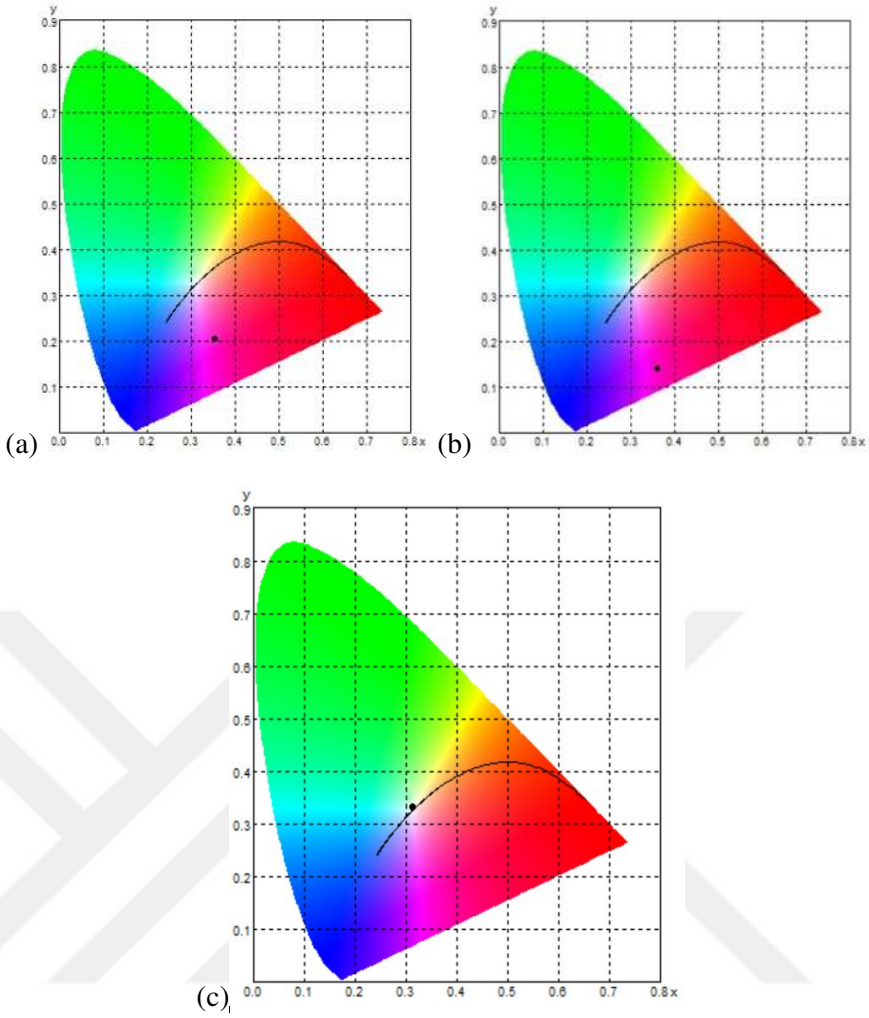


Figure 3.2. Chromatic diagram of x and y coordinate for the LED light combinations in the experiment a) G/2 b) G0 c) W control group.

3.1.2.2 Green Light Supplementation Experiment

Control groups (RB) were illuminated with 20 µmol m^{-2} s^{-1} in total containing only red and blue LED light with the light intensity of 12,3 and 7,99 20 µmol m^{-2} s^{-1} respectively. The ratio of RB light intensity for the control group was 77 and 16.4 respectively. In the experimental groups, green light intensity was increased as a fraction of red light.

The first experimental groups (G10) were illuminated with 20 µmol m^{-2} s^{-1} in total containing red, green, the blue LED light intensities of 10,7; 1,04; 8,3 µmol m^{-2} s^{-1} respectively. The ratio of Red and Blue light was kept as in white light condition

and green light intensity were increased to 10 % comparative to control groups of (RB). The ratio of RGB light intensity for the first experimental groups was 66/18/15 respectively.

The second experimental groups (G20) were illuminated with 20 µmol m^{-2} s^{-1} in total containing red, green, blue LED light intensities of 9,9; 1,63; 8,5 µmol m^{-2} s^{-1} respectively. The ratio of red, green and blue light intensities was kept as in white light condition and green light intensity were approximately increased to 20 % comparative to control groups (RB). The ratio of RGB light intensity for G20 was 59; 26,4; 14,7 respectively. The interface of chromatic diagram with x and y coordinate values for the LED light combination for all treatment in the second experiment were given in Figure 3.3(a,b,c).

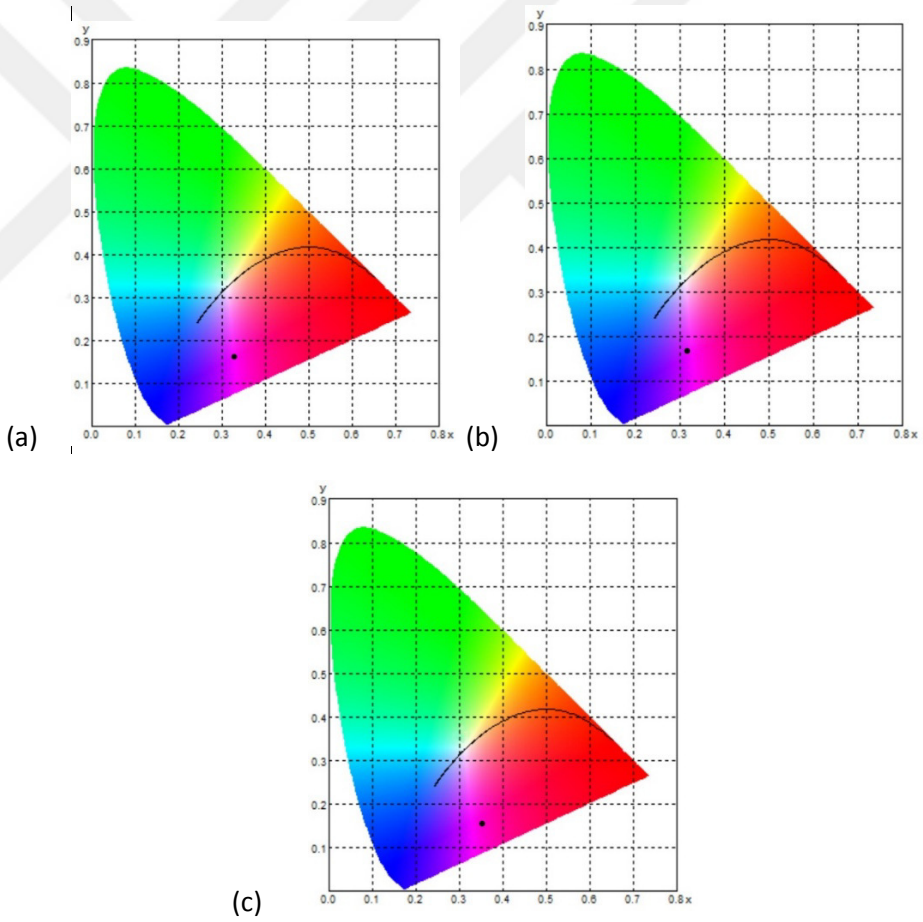


Figure 3.3. Chromatic diagram with x and y coordinate values for the LED light in green light supplementation experiment ; a) G10 (%10 green light supplementation); b) G20 (%20 green light supplementation); c) RB (Red and Blue Light).

3.2 Physiological Analysis

3.2.1 Determination of Cell Count And Biomass

For the cell count, 1 ml *H. pluvialis* sample was taken and added 15 µl Lugol for long-term preservation and stabilization of moving cells. 10 µl sample which was fixed with Lugol had been counted by Neubauer (Marienfeld Germany, 0,0025mm^2). This process was repeated at regular intervals by using a light microscope (Motic BA310E). And counted samples were preserved in the refrigerator.

For the determination of biomass, 2ml *H. pluvialis* was taken and put in the quartz cuvette for measuring in the spectrophotometer. This step was repeated at regular intervals by UV-6300PC Double Beam Spectrophotometer. 2 ml sample was mixed by pipetting, and measured spectrophotometrically at 750nm.

3.2.2 Estimation of Chl-a

2 ml Haematococcus pluvials samples were taken into 2 ml Eppendorf tubes and removed supernatant after samples centrifugated at 6000 x rpm for 5 minutes. 2 ml DMSO added into the pellet. These samples were incubated at 75^{o}C for 15 minutes. After the incubation, samples were centrifugated at 6000 x rpm for 5 minutes. 1 ml of the subsamples formed after centrifugation were taken into 2 ml Eppendorf tubes and added 1 ml DMSO. Prepare a blank cuvette filled with DMSO. After the sample for measurement of blank, all samples were measured spectrophotometrically at 665 and 720nm. Chl-a concentrations were calculated according to the formula:

$$\text{Chl-a} = 12{,}9447 * (665\ \text{nm} - 720\ \text{nm}) * \text{Dilution factor}$$

3.2.3 Measurement of Dry Weight

When these step had finished, samples were taken for measuring dry weight. Taking 40 ml *H. pluvialis* culture had sieved from 0,45 µm Whatman preweighted membrane filters. Then they were dried at -80 ºC overnight.

3.2.4 Genetic Sampling

Samples for total RNA isolation were taken at regular intervals. Algal cultures between 2 to 10 mL were centrifuged at 5500 rpm x 5 minutes and washed with PBS solution (Table 3.2). The aim of using PBS solution to maintain pH and osmotic balance. So, collected cells were maintained the short term in a viable condition. Collected samples were stored at -80 ºC until total RNA isolation.

Table 3.2. For 1L of PBS (Phosphate Buffered Saline, pH:7.4).

Compounds	Amount of compounds	Concentration
NaCl (mw: 58.4 g/mol)	8 g	0.137 M
KCl (mw: 74.551 g/mol)	200 mg	0.0027 M
Na_2HPO_4 (mw: 141.96 g/mol)	1.44 g	0.01 M
KH_2PO_4 (mw: 136.086 g/mol)	240 mg	0.0018 M

3.3 Molecular Analysis

3.3.1 Total RNA Isolation

Glass beads made from glass Pasteur pipette were sterilized by autoclaving and used for sample homogenizing. Additionally, before the RNA extraction, glass beads and all materials were exposed in UV rays for removing possible contamination.

Total RNA was extracted using the Trizol® extraction method (Invitrogen, USA) according to the manufacturer's protocol. Algae samples preserved at -80°C were homogenized by vortexing using glass beads into 2 mL tubes including 100 µl

TRIzol reagent for 2 min and immersed into liquid nitrogen immediately. The procedure was repeated 4 times for intense homogenization and to avoid RNA degradation. Then an additional 500µl TRIzol was added into the homogenized samples and incubated for 20 min at room temperature for complete dissociation of algae cells. After sample homogenization in 0.6 mL of Trizol, total RNA was precipitated with the addition of 0.5 mL of 100% isopropyl alcohol with subsequent RNA phase separation from chloroform at 20000xg centrifugation for 20 min at 4 ºC.

Precipitated RNA pellets were visible at the bottom of the tubes after centrifugation were washed 2 times with 75% cold ethanol. Following air-dried of RNA pellets, they are resuspended with 40 µl of nuclease-free water. Resuspended RNA solutions were incubated in the hot block at 55 ° C for 5 minutes. In order to eliminate potential DNA contamination, total RNA extractions were treated with a DNase treatment (TURBOTM Ambion Life Technologies, USA). The RNA quality and integrity were checked spectrophotometrically at 230-260-280 nm and by electrophoresis through a 1.5% agarose gel in TBE buffer (Table 3.3) following formamide denaturation. Total RNA samples were stored at -80° C until cDNA synthesis for qRT-PCR.

Table 3.3. Chemicals required to prepare 1L 10x TBE (Tris-borate-EDTA) buffer.

Compounds	**Weight**	**Concentration**
Tris base	108 g	890 mM
Boric acid	27.5 g	890 mM
0.5 M EDTA (solution) (ph:8.0)	40 ml	20 mM

3.3.2 cDNA Synthesis from RNA

For the synthesis of cDNA, Thermo RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, USA; Catalogue number: # K1622) with an oligo $(dT)_{18}$ primer was used according to manufacturing protocols. Each sample was prepared with and without reverse transcript as a positive and negative control of the samples respectively. Additionally, for each cDNA reaction, positive control with GAPDH genes provided by kit were prepared to check the success of the reactions. A no-template control was also prepared in order to check possible contamination of kit

content and primer sets. All the cDNA samples were preserved at -80 ºC until qPCR applications.

3.3.3 qRT-PCR

Four mRNA (RubisCo, $PTOX_2$, PSAB, PSBS,) were used in qPCR analyses. 18S ribosomal RNA (18S) were used as normalized control transcripts for mRNA. qPCR analyses were carried out on the BioRad CFX96 C1000 Touch Systems. Each reaction contained 10 or 5 ng template cDNA, 500 nM or 300 nM of each primer, and 5 µl of iTaq Universal SYBR Green Supermix (BioRad) in a final volume of 10 µl for mRNA. Reactions were conducted in triplicate, including negative controls, and all biological replicates were repeated twice. Cycling parameters for both mRNAs were 95 ºC for 30 sec followed by 40 cycles of 90ºC for 5 sec and 30 sec at 58ºC. Melt curves carried out at the end of the amplification cycle confirmed the absence of nonspecific amplification and primer dimers. A seven-point standard curve of a ten-fold dilution series was included in each run and used to calculate amplification efficiency for each primer set. Primer sequences and the reference transcripts were selected using Primer3Plus software (Andreas et al., 2007) and are given in Table 3.4 Primer specificity was confirmed using BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) against the *H. pluvialis* genome.

Table 3.4. List of used primer for gene expression in qPCR.

Gene	Access N	Primers (Forward and Reverse)	Amplification size	Primer size (bp)
18S-F	KU193764.1	CAAAGCAAGCCTACGCTCT	139	19bp
18S-R	KU193764.1	ATACGAATGCCCCCGACT	139	18 bp
RUBISCO-F	DQ178607.1	TAGGTGCTGCCGTGATTTTG	102	20bp
RUBISCO-R	DQ178607.1	AGGCAAACAGCTACTGCAAC	102	20bp
PTOX2-F	DQ485458.1	TTTGGACAGCAAAGCTTCGC	140	20bp
PTOX2-R	DQ485458.1	TTTTTGGCAAGGGTGAAGGG	140	20bp
PSAB-F	AB084365.1	TGCACAAAACCCTGATACCG	147	20bp
PSAB-R	AB084365.1	ACCGCAATTGCTAAGTGGTG	147	20bp
PSBS-F	DV203099.1	ACTATTGGCGAGATCCTGCTG	115	21bp
PSBS-R	DV203099.1	TGCCGTTGAACAGCACAATG	115	20bp

3.4 Statistical Analysis

All data values were checked as means (±SD). Statistical valuation of physiological and genetically was carried out with a one-way analysis of variance (ANOVA). The significance ($p \leq 0.05$) of the variables studied were adjusted by post-hoc Tukey HSD.

4. RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Green Light Substraction

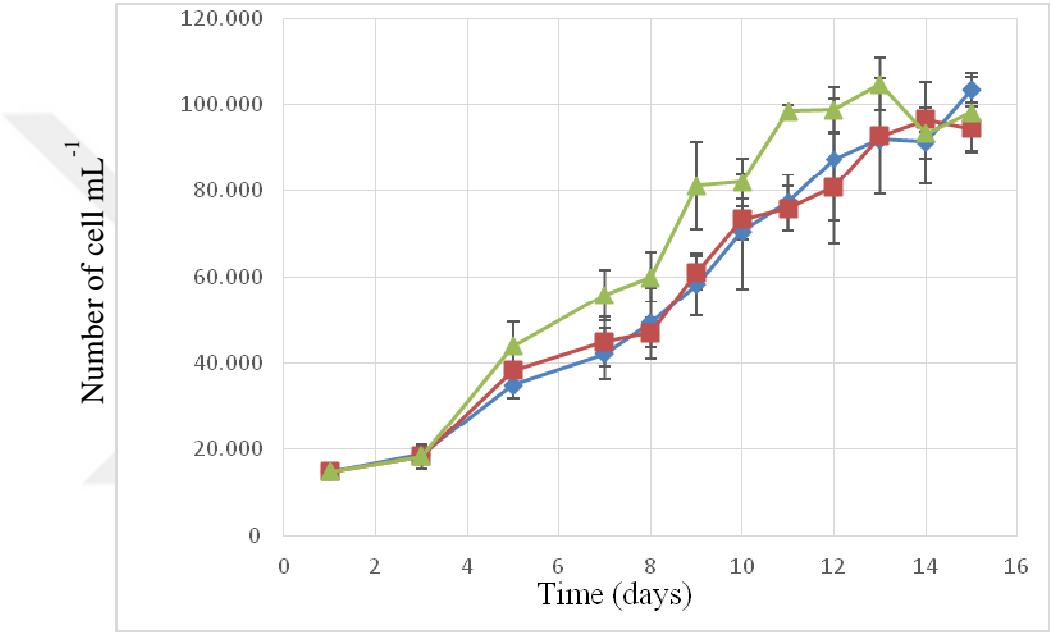


Figure 4.1. Growth curve of *H. pluvialis* in experimental treatments. Control groups (W): White Light (♦); G/2 (■): 50 % green light subtraction; G0 (▲): 96 % green light subtraction.

The growth curve of *H. pluvialis* as regard with the number of cells per mL under 18 hours light / 6 hours dark illumination periods, 50 L h^{-1} aerations supplemented with 1.5% CO_2, 20 µmol photons m^{-2} s^{-1} of total light intensity with different green light regimes throughout 15 days of the experiment (Figure 4.1)

White light was used as control treatment with R: G: B ratio of 31.3: 61.1: 4.6 including 8.31; 6.75: 5.39 µmol photon m^{-2} s^{-1} light intensity respectively. G/2 and G0 treatments were prepared by keeping red and blue and light ratios (approximately 6:1) the same as control groups of white light and subtraction of green light as an advance of red light in the ratio of 50 % and 96 % respectively.

Total light intensity was applied as 20 µmol photons m^{-2} s^{-1} for all the treatments. Therefore, light combination of G/2 and G0 treatments have R: G: B ratio of 59.1: 32.5: 8.4 and 83.3: 4.1: 12.6 with the light intensities of 11.61: 2.51: 6.82 and 12.8:0.2:7.91 µmol photon m^{-2} s^{-1} respectively.

Table 4.1. Comparison of the growth rate of H. pluvialis as regard with the number of cell per µL in the green light subtraction of 11^{th} days of the incubation period.

Treatment Pairs	Q	p
W – G/2	0.5372	0.900
W – G0	7.5807	**0.004**
G/2 – G0	8.1179	**0.003**

* p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

According to results given in Figure 4.1, G0 shows faster and higher growth rate than G/2 and W treatments. 96% of green light subtraction as an advance of red light in G0 treatments have a stationary state at 11^{th} days of the experiment that 5 days earlier than W and G/2 treatments. G0 have statistically higher growth rate at 11^{th} days of experiments than W and G/2 treatments. (Table 4.1) (One way ANOVA, F: P<0.001). 50% of green light subtraction as an advance of red light in G/2 treatment did not show significant differentiation between W treatments at 11^{th} days of the green light subtraction (One way ANOVA, F: P>0.05). Cell numbers in 11^{th} days were $9.85x\ 10^4 \pm 1.3x\ 10^3$, $7.58x\ 10^4 \pm 5.2x\ 10^3$, $7.73x10^4 \pm 6.4x\ 10^3$ observed in G0, G/2 and W treatments at 11^{th} day of experiment respectively. Maximum cell numbers were $1.05x\ 10^5 \pm 6.1x\ 10^3$, $9.27x\ 10^4 \pm 1.32x\ 10^3$, $9.2x10^4 \pm 4x\ 10^3$ observed in G0, G/2 and W treatments at 13^{th} day of experiment respectively.

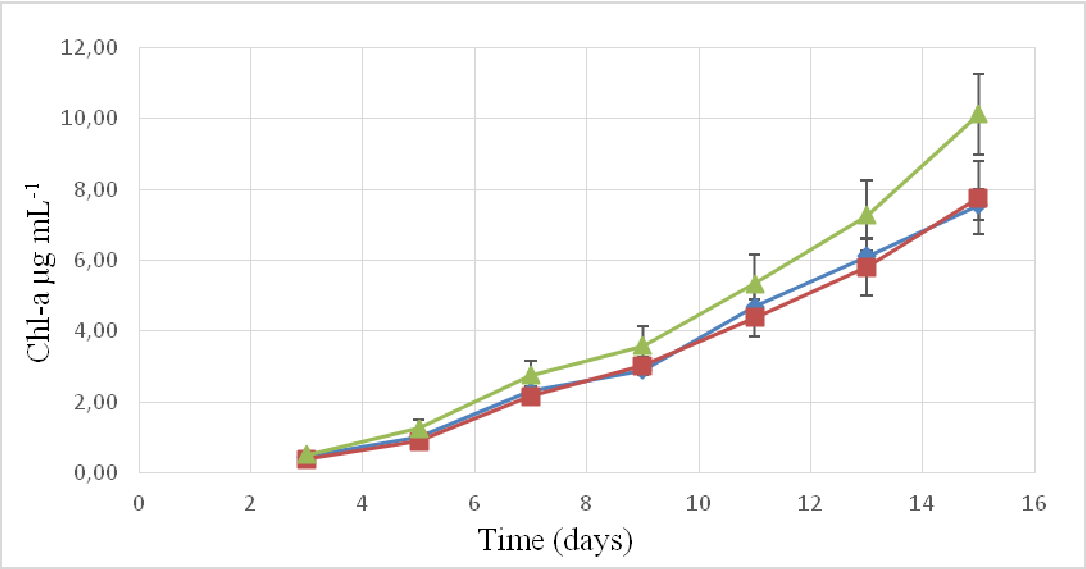


Figure 4.2. Chlorophyll-a concentrations of *H. pluvialis* in experimental treatments. Control groups (W): White Light (♦); G/2 (■): 50 % green light subtraction; G0 (▲): 96 % green light subtraction.

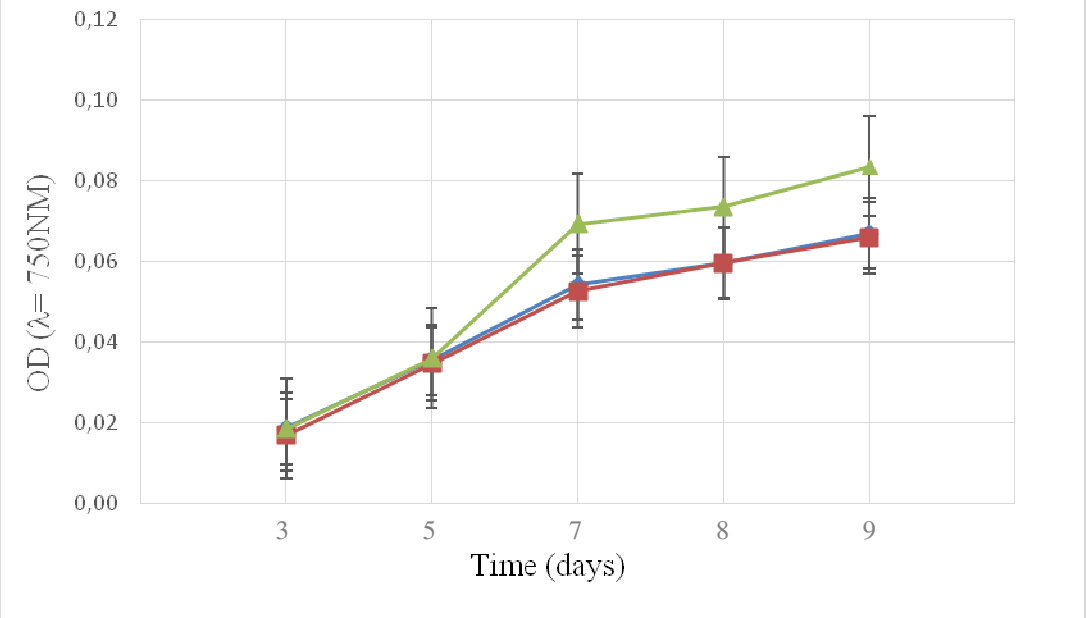


Figure 4.3. Optical Density (OD) at 750 nm for *H. pluvialis* in experimental treatments. Control groups (W): White Light (♦); G/2 (■): 50 % green light subtraction; G0 (▲): 96 % green light subtraction.

Changes of chl-a concentration and OD values measured at 750 nm spectrophotometrically throughout the green light subtraction, respectively (A.4, A.5). Chl-a and OD values are complementary with growth rates that G0 has higher values than W and G/2 treatments (Figure 4.2, 4.3). Chl-a concentration and OD values of G0 have departed from G/2 and W treatment at the 5th day of experiments that are consistent with the growth rate in Figure 4.1. The highest chlorophyll values

of the treatments are observed at 15th day of the experiment in the values of 7.57 ± 0.43; 7.77 ± 1.04 and 9.40± 1.14 mg ml^{-1} for W, G/2 and G0 respectively. The OD values at 5th day of the experiment at 750 nm for W, G/2 and G0 are 0.07, 0.07 and 0.08 respectively (Figure 4.3).

Table 4.2. Comparison of chl-a concentrations in green light subtraction experiment at 15th days.

Treatment Pairs	Q	p
W – G/2	0.4019	0.900
W – G0	4.6019	**0.047**
G/2 – G0	4.2000	0.070

* p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

Table 4.3. Comparison of OD750nm value in green light subtraction experiment at 9th days.

Treatment Pairs	Q	p
W – G/2	0.2746	0.900
W – G0	4.5771	**0.041**
G/2 – G0	4.8517	**0.032**

* p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

Chl-a and OD values of G0 treatments show statistically significant differences than W and G/2 treatments (P<0.05) except for between G0 and G/2 in Chl-a concentrations that is moderately significant (Table 4.2, 4.3).

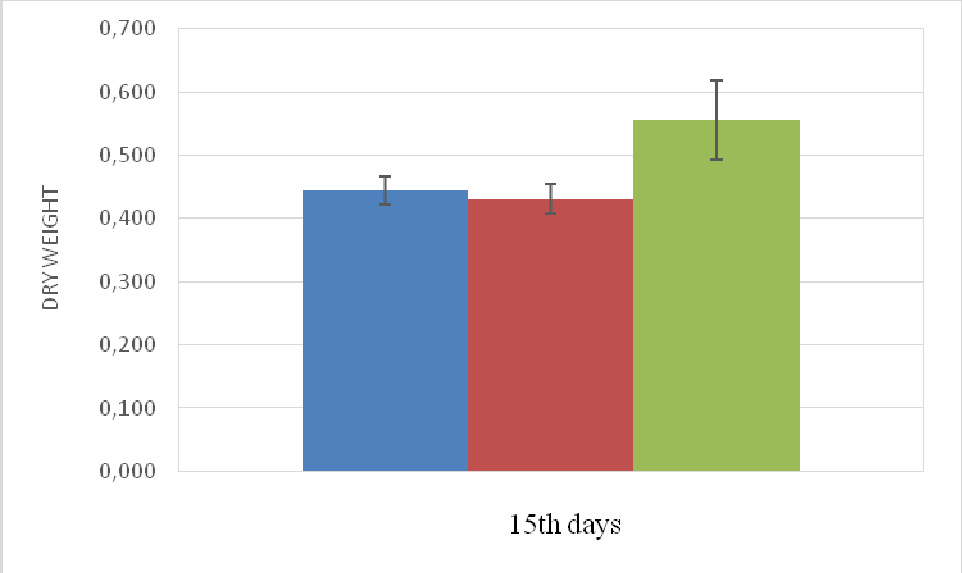


Figure 4.4. The dry weight (DW) of *H. pluvialis* at the end of the experiment. Control groups (W): White Light (♦); G/2 (■): 50% green light subtraction; G0 (▲): 96% green light subtraction.

According to results given, G0 shows higher dry weight than G/2 and W treatments (Figure 4.4).

Table 4.4. Comparison of DW in green light subtraction at the end of experiment.

Treatment Pairs	Q	p
W – G/2	0.5106	0.900
W – G0	4.5955	**0.040**
G/2 – G0	5.1061	**0.026**

*p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

A statistically significant difference was observed between G0 and other groups when compared statistically (One way ANOVA, F: p<0.05). There was no statistically significant difference between W and G/2 treatments (One way ANOVA, F: P>0.05) (Table 4.4).

4.1.2 Green Light Supplementation

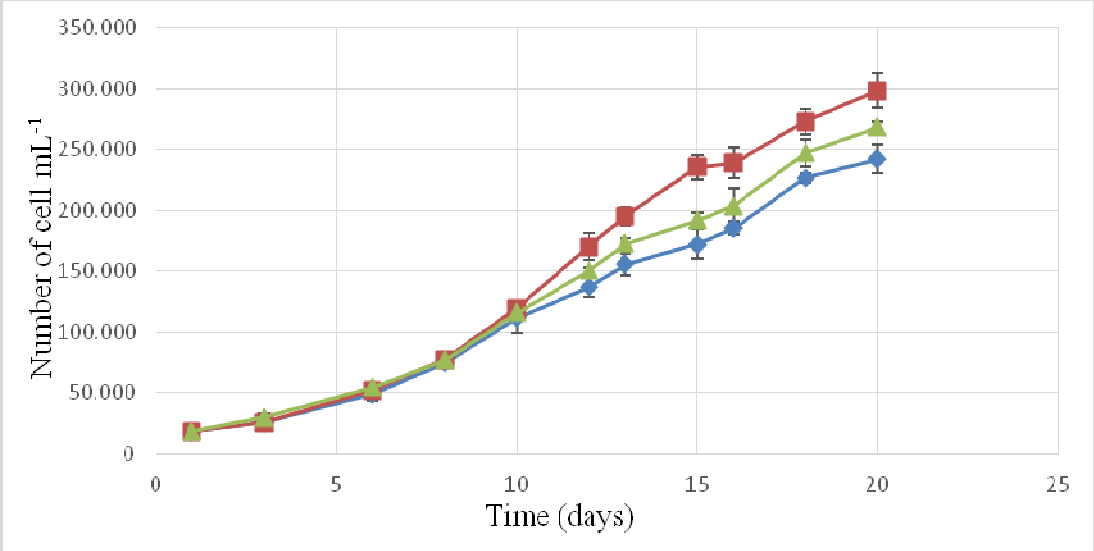


Figure 4.5. Growth curve of *H. pluvialis* in experimental treatments. Control groups (RB) Red-Blue Light (♦); G10 (■): 10% green light supplementation; G20 (▲): 20% green light supplementation.

This figure shows growth curve as regard with the number of cells *of H. pluvialis* per mL under 18 hours light / 6 hours dark illumination periods, 50 L h^{-1} aerations supplemented with 1.5 CO_2, 20 µmol photons m^{-2} s^{-1} of total light intensity with different green light supplementations throughout 20 days of experiments. Red-

blue (RB) light was used as control treatment having R:G:B ratio of 77: 6.6: 16.4 including 12,3: 0,007: 7,99 µmol photon m^{-2} s^{-1} light intensity respectively. G10 and G20 treatments were prepared by keeping red and blue and light ratios the same as control groups of Red-Blue light (approximately in the ratio of 6:1) and supplementation of green light as the extraction of red light intensity in the ratio of 10 % and 20 % respectively. Therefore the light combination of G10 and G20 treatments have R:G:B ratio of 66: 18: 15 and 10,7: 1,04: 8,3 with the light intensities of 59: 26.4: 14.7 and 9.9: 1,63: 8,5µmol photon m^{-2} s^{-1} respectively.

All the treatments show the same growth rate in the first 10^{th} day of the experiment. From that point of the experimental day, G10 and G20 show a consistent divergence from the control treatment of RB (Figure 4.5).

Table 4.5. Comparison of the growth rate of RB, G10 and G20 treatments at 20^{th} days of green light supplementation experiment.

Treatment Pairs	Q	p
RB – G10	8.7400	**0.004**
RB – G20	4.5497	**0.052**
G10 – G20	4.6707	**0.047**

*p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

10% and 20 green light supplementation in G10 and G20 show statistically significant higher growth rate than RB control groups at the end of the experiment (Table 4.5). Maximum cell number was measured as $2.42x10^5 \pm 1.1x10^4$; $2.98x10^5 \pm 1.4x10^4$ and $2.68x10^5 \pm 4.7x10^3$ in RB, G10 and G20 treatments at 20^{th} day of the experiment respectively (A.3).

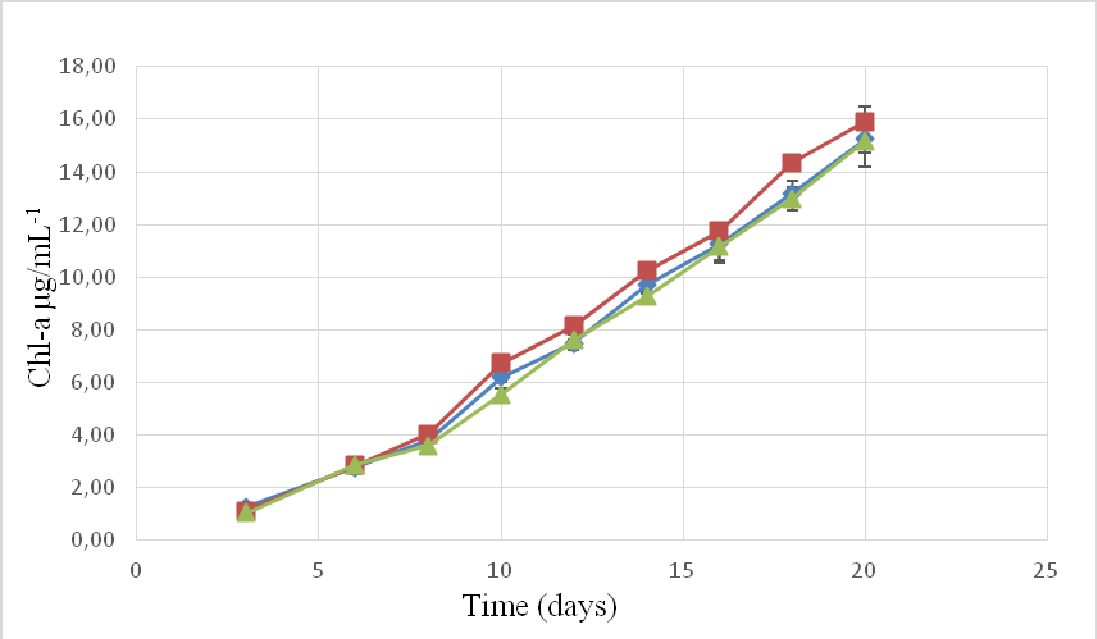


Figure 4.6. Chlorophyll-a concentrations of *H. pluvialis* in second experimental treatments. Control groups (RB) Red-Blue Light (♦); G10 (■): 10% green light supplementation; G20 (▲): 20% green light supplementation.

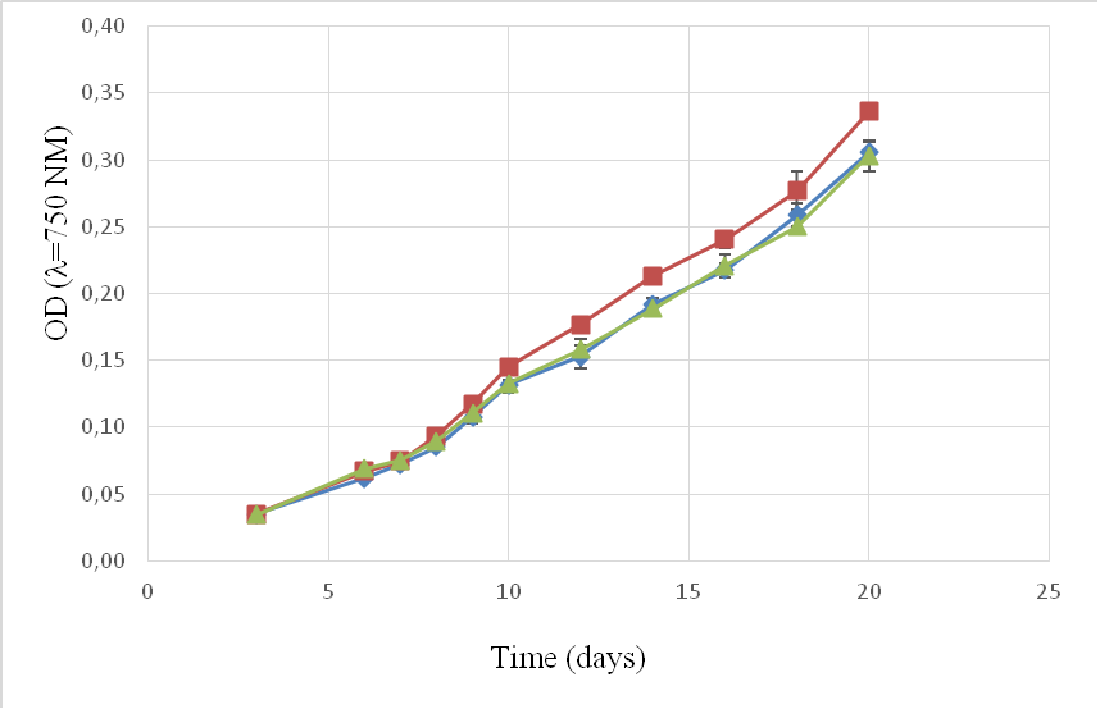


Figure 4.7. OD750 nm value of *H. pluvialis* in experimental treatments. Control groups (RB) Red-Blue Light (♦); G10 (■): 10% green light supplementation; G20 (▲): 20% green light supplementation.

Changes of chl-a concentration and OD values measured at 750 nm spectrophotometrically throughout the experiment respectively. Chl-a concentration and OD values are consistent with growth rate results that G10 has the highest values of these measurements (Figure 4.6, 4.7). There are statistically significant differences between control and G10 - G20 treatments (Table 4.6, 4.7). Chl-a concentration at the end of the experiments are 15,25 $\mu g/mL^{-1}$ ± 0,53 μgmL^{-1} ; 15,92 $\mu g\ mL^{-1}$ ± 0,55 $\mu g\ mL^{-1}$; 15,18 $\mu g\ mL^{-1}$ ± 1,01 $\mu g/mL^{-1}$ in RB, G10 and G20 treatments at 20th day of the experiment respectively. OD values at the end of the experiments are 0,31 ± 0,01; 0,34 ± 0,01 ; 0,30 ± 0,01 in RB, G10 and G20 treatments at 20th day of the experiment respectively.

Table 4.6. Comparison of chlorophyll-a concentration of green light supplementation experiment at 20th days.

Treatment Pairs	Q	p
RB – G10	5.373	**0.021**
RB – G20	0.9058	0.791
G10 – G20	6.2792	**0.010**

*p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

Table 4.7. Comparison of OD750 nm value of green light supplementation experiment at 20th days.

Treatment Pairs	Q	p
RB – G10	4.8080	**0.043**
RB – G20	0.4887	0.900
G10 – G20	5.2450	**0.031**

*p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

According to results, G10 shows a higher amount of dry weight than RB and G20 treatments (Figure 4.8). The DW for RB, G10 and G20, respectively; 0.73; 0.80; 0.65g. There was no statistically significant difference between RB, G10 and G20 treatment (One way ANOVA, F: P>0.05) (Table 4.8).

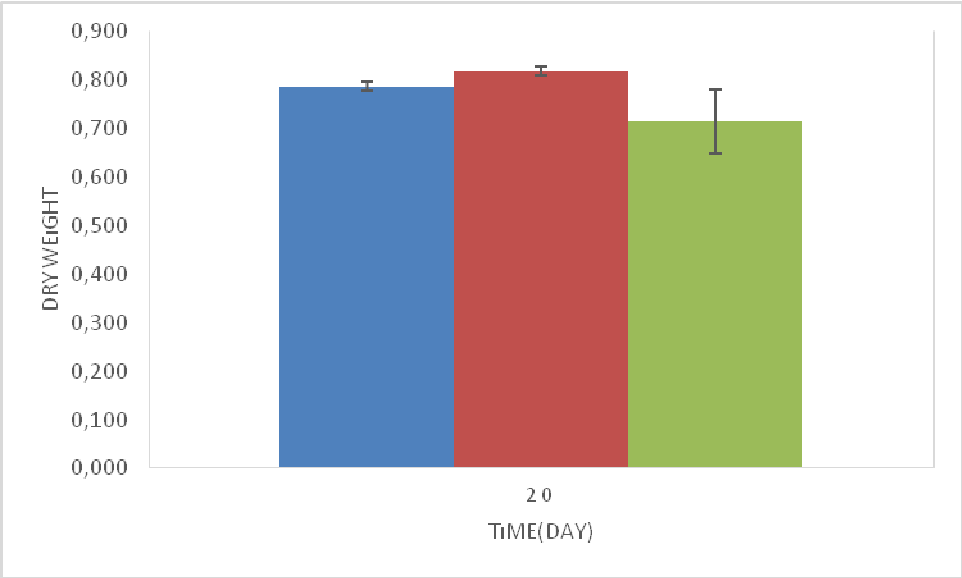


Figure 4.8. The dry weight (DW) of *H. pluvialis* in green light supplementation experiment at 20th days. Control groups (RB) Red-Blue Light (♦); G10 (■): 10 % green light supplementation; G20 (▲): 20 % green light supplementation.

Table 4.8. Comparison of dry weight in green light supplementation experiment at 20th days.

Treatment Pairs	Q	p
RB – G10	1.1537	0.699
RB – G20	1.5332	0.557
G10 – G20	2.6869	0.219

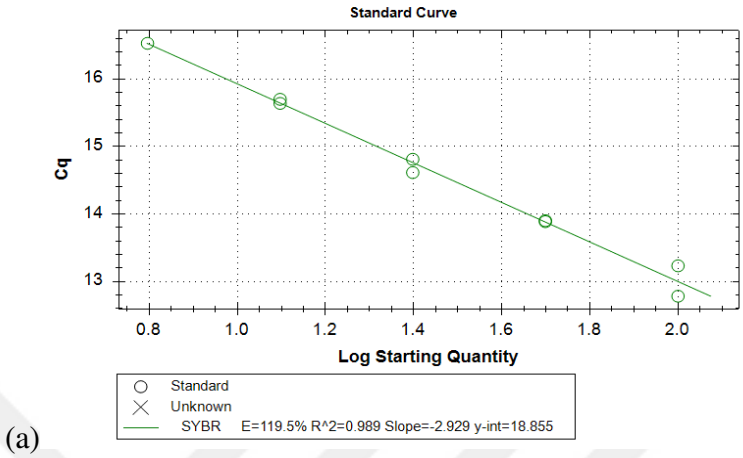
*p<0,05 statistical significance, (One Way ANOVA, Tukey HSD test)

4.1.3 Transcriptional Expression Differentiations Relative mRNA Expression

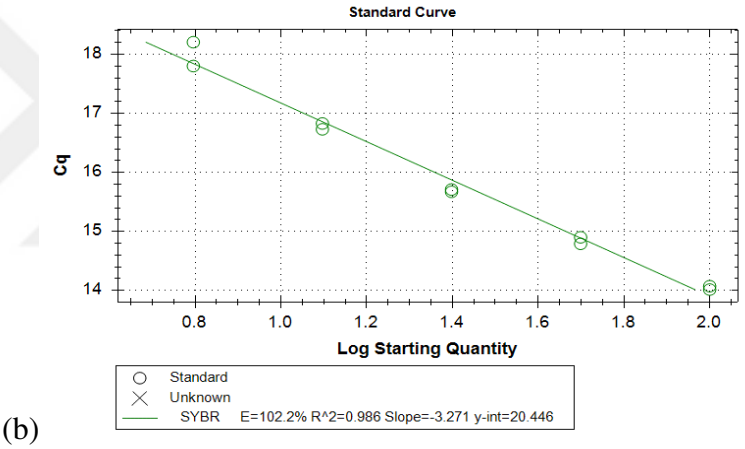
In the present study, transcriptional changes of 4 mRNA transcript (RubisCo, $PTOX_2$, Chloroplast PsaB and PsbS) were analysed. A qPCR approach was carried out on RNA isolated samples exposed to 2 different green light supplementation and RB light regimes for 3 different time point throughout the growth of *H. pluvialis* at initial (t0), 5th (t5) and 20th (t20) day of the experiments. For data normalization, 18S ribosomal mRNA transcript was used as several reports have indicated stability under similar experimental conditions.

Five-point standard curve of a ten-fold dilution series for each qPCR run that was used to calculate amplification efficiency for each primer sets (Figure 4.9).

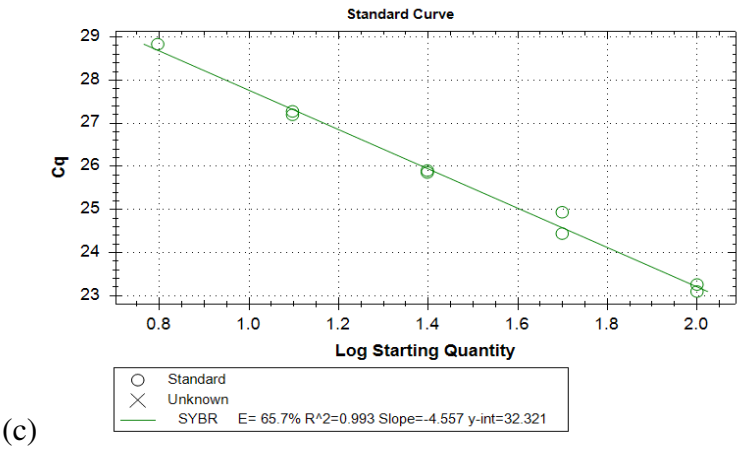
Standard curve analyses show R^2 values between % 88 to 99 efficiency. Primer sequences were designed using Primer 3 Plus software (Andreas et al., 2007) and were confirmed using BLASTn against *H. pluvialis* genome in NCBI.



(a)



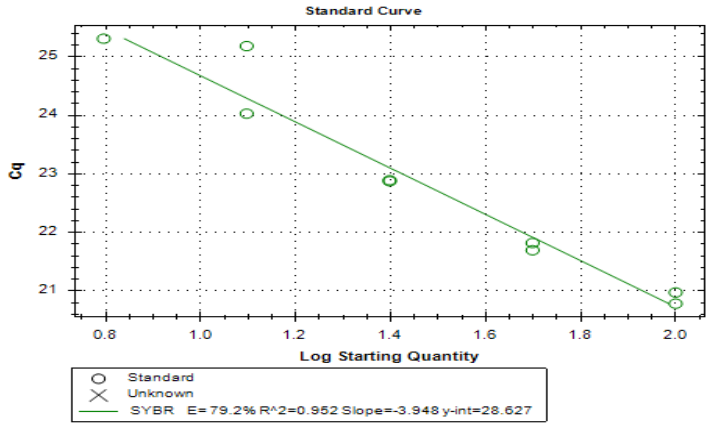
(b)



(c)

Figure 4.9. Standart curve of a ten-fold dilution series based on five concentration point for each qPCR primers a)18S, b) RubisCo, c) $PTOX_2$

(a)



(b)

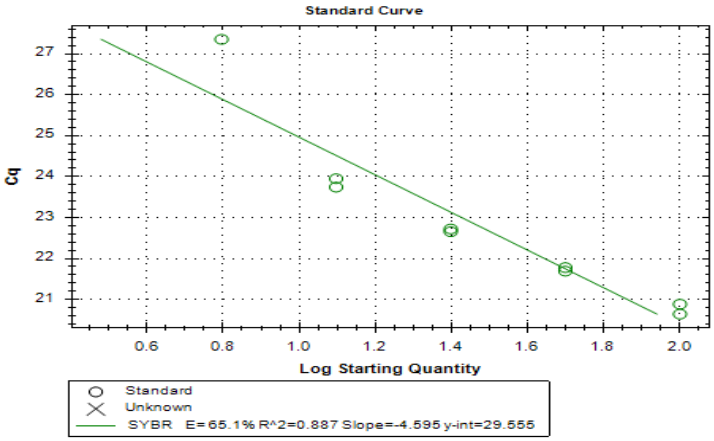


Figure 4.9 (continue). Standard curve of a ten-fold dilution series based on five concentration point for each qPCR primers a)18S, b) RubisCo, c) $PTOX_2$ d) PsbS, e) PsaB.

The melting curves carried out at the end of the amplification cycle to confirm if there are nonspecific amplification and primer dimers (Figure 4.10). None of the primer sets showed non-specific amplification peak that confirms the success of amplification specificity for primers sets of analyzed genes.

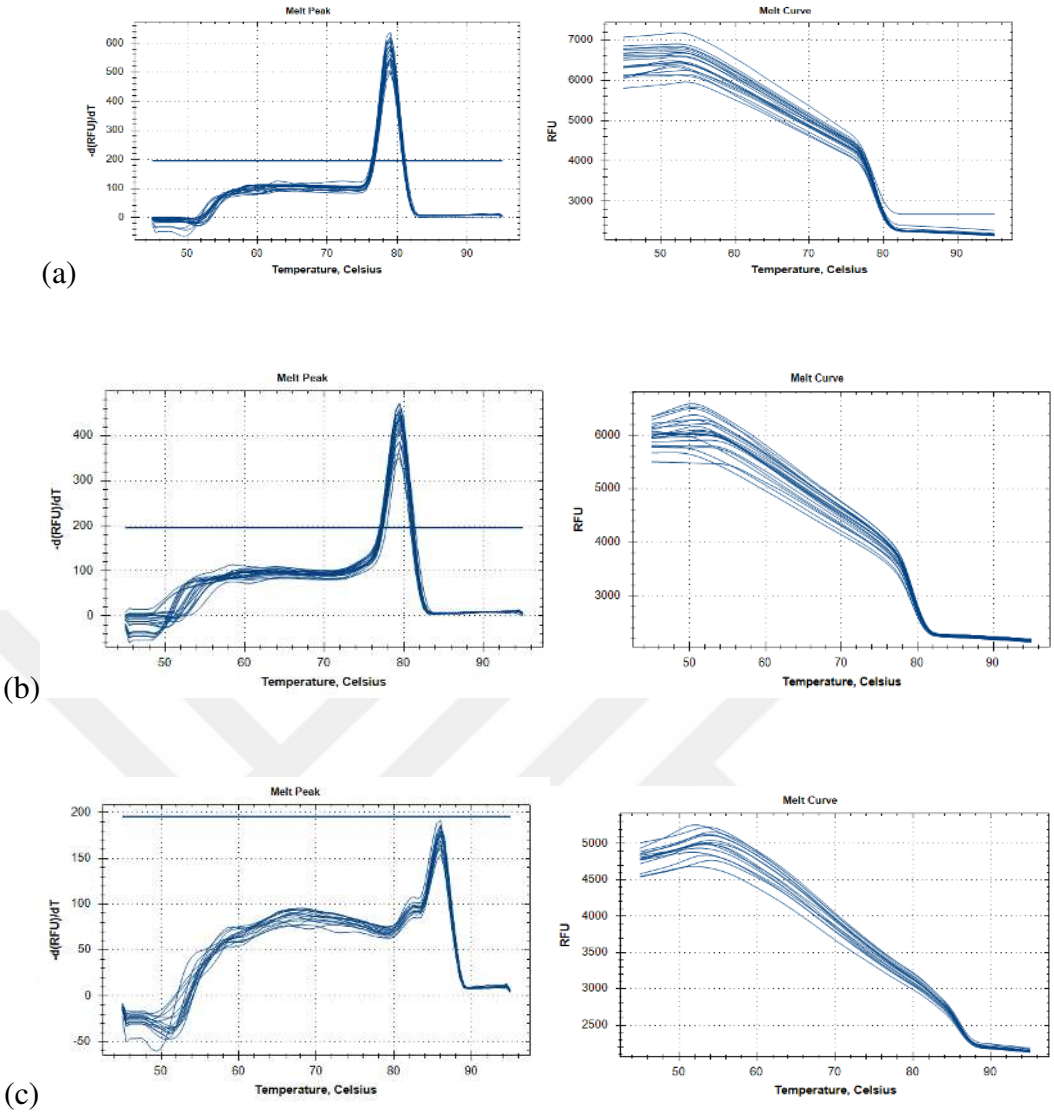
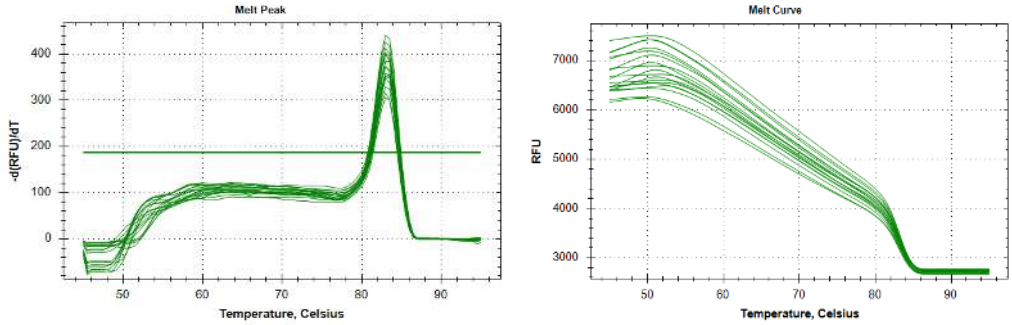


Figure 12. Melting curve and peak grafic of the studied qPCR primers. a) 18S, b) RubisCo, c) $PTOX_2$

(a)



(b)

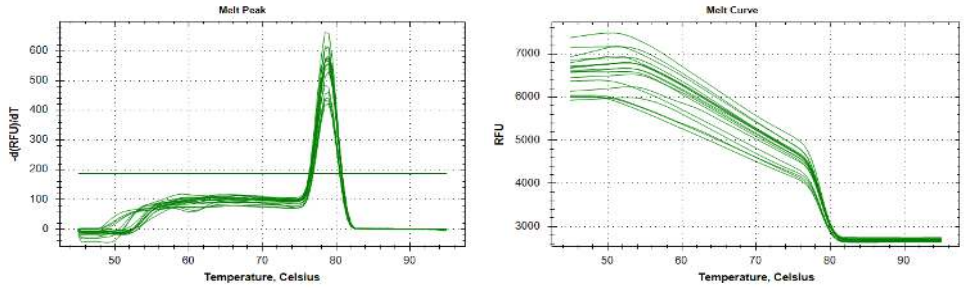


Figure 4.10 (continue). Melting curve and peak grafic of the studied qPCR primers. a) 18S, b) RubisCo, c) $PTOX_2$ d) PsbS, e) PsaB.

Fold-change differences between exposure times of green light supplementation were calculated based on the expression of initial time point- t_0 using ΔCt method and normalized to the expression of ribosomal 18S gene transcripts. Data having between 85% and 99% of amplification efficiency were selected to analyse.

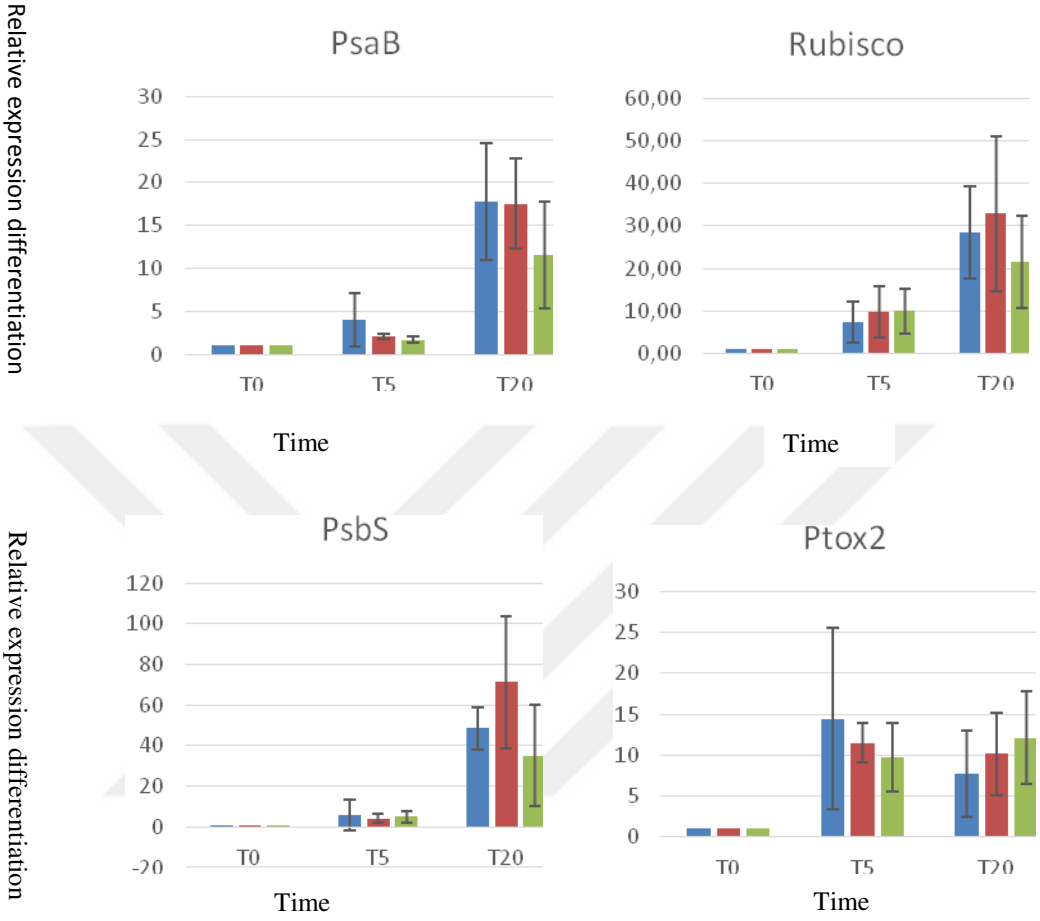


Figure 4.11. Expression differentiation of RubisCo, $PTOX_2$, chloroplast PsaB and PsbS genes for G0 (●), G10 (●) and G20 (●) treatments at initial (t_0), 5th (t_5) and 20th days (t_{20}) of the experiment.

Relative expression differentiation of RubisCo, $PTOX_2$, chloroplast PsaB and PsbS genes for G0, G10 and G20 treatments at initial (t_0), 5th day (t_5) and 20th day (t_{20}) of experiments (Figure 4.11).

Relative expression of studied of genes shows upregulation from t_0, to t_{20} of the experient time points. Expression differentiation variate between 3.6 to 12.8, 1.7 to 3.5, 1.8 to 6.2 and 6.6 to 22.2 at t_5; 20.3 to 40.8, 32.1 to 59.6, 12.9 to 22.6 and 4.1 to 11.5 at t_{20} for RibusCo, chloroplast PsbS, chloroplast PsaB and $PTOX_2$ respectively. All the treatments show statistically significant differentiation in

transcriptional expression between time points of t_0, t_5, and t_{20} (One way ANOVA $P<0.001$) except for $PTOX_2$ between t_5, and t_{20} (One way ANOVA $P>0.05$).

Comparison of G0, G10 and G20 treatments at t_{20} to assess green light effects on the transcriptional expression of studied genes show that chloroplast PsbS gene showed statistically significant fold change differentiation in G10 treatment than G0 and G20 (One way ANOVA $P>0.05$). The rest of the genes did not show statistical differences between treatments at t_{20} as regard with fold change expression differentiation. Moreover, no genes show statistically expression differentiation between treatments at t_5 (One way ANOVA $P>0.05$).

4.2 Discussion

In the last two decades, the physiological, molecular and biotechnological examination of microalgae has become a quite popular topic because of its economic value. Many different species of microalgae have been investigated based on active molecular components and metabolites they synthesize. *H. pluvialis* with its high antioxidant properties is located among the most researched species recently. Researches have mainly focusing on factors such as light, nutrient composition, carbon assimilations rates and induction types to improve the growth and astaxanthin production of *H. pluvialis*. Among these factors, light quality and quantity have a central role in microalgae production. In large scale productions, research is carried out on the light source to reduce the cost and get high efficiency in a short time (Kim et al., 2004).

4.2.1 Effects of R-G-B LED Light Spectrums on Growth Parameters of *H. pluvialis*

Recent studies rely on LED light have given promising results in both microalgae growth rate and secondary metabolite activity comparatively other light sources such as fluorescence and halogen light sources. Due to the narrower spectrum of light, low electricity consumption and flexible application capacity, LED light has been extensively used in microalgae production. Moreover, LED light

applications to provide researchers to combine different light spectrum in different intensity in their research. (Katsuda et al., 2004).

In studies conducted on plants species, it has been observed that monochromatic and combination blue and red light have an important effect on plant development. Results of these studies show that the mixture of blue and red LED spectrums is more effective in increasing the growth parameters of algae and plant species compared to the monochromatic application of red and blue light (Nhut et al., 2000; Li et al., 2010; Shin et al., 2008). Moreover, blue and red light has opposite effects on chlorophyll concentration in plant species. While monochromatic blue light propagates to increase chlorophyll synthesis, monochromatic red LED light result in a decrease in comparison with white light sources (Tanaka et al., 1998; Li et al., 2010).

Red light perceived by the light photoreceptor 'phytochromes' (Bussell and Kehoe, 2013; Kianianmomeni and Hallmann, 2014) accelerates the cell cycle of many microalgae and induces cells to grow (Blanken et al., 2013; Chen et al., 2010). Blue light is perceived by cryptochromes which is one of the photoreceptors and affects gene expression and various metabolic pathways through cryptochrome in algae and plant (Lin, 2002; Beel et al., 2012). Blue light results in ROS formation in algae and plant cells because of the higher energy which is higher than the energy requirements for photosynthesis. Therefore, algae and plants are induced to accumulate photoprotective pigments (xanthophyll, astaxanthin, etc.) to protect the photosynthesis mechanism against ROS (Jahns and Holzwarth, 2012).

Studies regarding the physiological effects of light spectrums on algae and plant species are concentrated on monochromatic or specific ratios of blue and red lights. It has been reported by several comprehensive studies that the most effective mixing ratio of red and blue light is 3:1 for the most effective growth rate of green algae species including *H. pluvialis* (Katsuda et al., 2004; Kobayashi et al., 1992a; Koc et al., 2013; Suyono et al., 2015). The absorption rate of red and blue light by chlorophyll pigments is higher than another light spectrum than green light (Kim et al., 2004). Therefore, the green light has received very limited attention in algae and plant studies. Only a few studies have mentioned about the effects of monochromatic

green light on algae and plant growth parameters (Olle and Virsile, 2013; Kim et al., 2004; Terashima et al., 2009).

In the study of Gaytán-Luna et al. (2016) white, green and red light sources were compared regarding biomass and chlorophyll concentration in *Chlamydomonas reindartii*. Red and green light illumination results in 5- and 3-times higher chlorophyll concentration than white light sources respectively. The highest growth rate was achieved in red light illumination, while the minimum growth rate with shorter time duration to rich exponential growth phase was observed in green light sources. It is concluded that a lower absorption rate of green light might result in higher chlorophyll concentration and lower growth rate in *C. reindartii*. Another study conducted with *Nannochloropsis sp*. by Vadiveloo et al. (2015), resulted in a non-effective growth rate in green light illumination. In the study of Blair et al. (2014), *Chlorella vulgaris* culture had higher growth rate with blue light illumination compared to green, red and white light. These studies indicate that the best performance of growth rate varies according to light spectral quality of blue and red-light sources and suppressed by the monochromatic green light condition. Likewise, Kendirlioglu and Cetin (2017) reported limited cell growth under monochromatic green light supply in *Chlorella vulgaris*, while the highest growth in red light. Moreover, the different combination of red and blue light such as blue-purple LED also result in higher growth rate than white light, and lowest growth rate in green light in *H. pluvialis* (Katsuda et.al., 2004). Under the light of this literature knowledge, it is concluded that monochromatic green light results in a very low growth rate and suppress cell division in both algae and plant species.

Beside monochromatic green light applications, only a couple of study addressing the different combination of green light with red and blue light spectrums were recorded. Results of these limited number of studies pointed on an interesting effect of green light on terrestrial plant species. Kim et al. (2004) applied a different fraction of green light with a combination of red and blue light on lettuce. They used red and blue light combination at 3:1 ration with supplementation green light at ratios from 0 to 51 % of total illumination. Results were surprising that 24 % of green light supplementation results in an increase in growth rate and dry weight of lettuce

production. They recorded a lower growth rate at both upper and lower percentage of that specific green light supplementation rate of % 24 than control groups. This record is contradicted with the previous study referring to the minor regulatory roles of green light on photosynthetic species.

Although, no comprehensive studies is explaining the function and roles of green light, it has been speculated that green light might regulate photosynthesis, physiological responses and/or phenotypical traits in plant species (Smith et al., 2017). Indeed, depending on species, around 10 -50 % of green light is reflected, the rest of them is absorbed by photosynthetic pigments (Terashima et al., 2009). The green light has higher penetration rates into the mesophyll layers of plant leaves therefore, it may be logical to conclude that green light may have additive roles within the photochemical cycle of plant cells. Moreover, the green light is able to penetrate a deeper part of the forest where red and blue light spectrum are not available enough for photosynthesis. Plant species located lower vertical level of the forest may have evolved to use green light differently than the canopy. Chlorophyll a and b mainly absorb red and blue light spectrum (λ600-700 nm and λ400–500 nm respectively) but not significantly absorb in green light regions (λ500–600 nm). However, other pigments conjugated with chlorophyll such as flavonoids, anthocyanins and carotenoids are able to absorb green light spectrums (Smith et al., 2017). These pigments may have an accessory role in light-harvesting to pass the light energy to chlorophyll pigments for photosynthesis (Hopkins and Hüner, 2009; Holleboom and Walla, 2014). However, such conclusions refer only terrestrial plant, but not for aquatic algal species.

The present study is the first research that aims to investigate dose-dependent green light effects in algae using physiological and molecular approaches. Our results support the study performed by Kim et al. (2004). 10 % of green light supplementation increases the growth rate of *H. pluvialis* along with 3:1 ratio of red and blue light source. This alteration in growth parameters of cell number, chl-a concentration and optical cell density is also found in relative gene expression of *H. pluvialis* in RuBisCo (related with carbon assimilation) and PsbS genes (regulatory gene of electron transfer in PSI).

The additive effects of 10% green light supplementation are observed in the early exponential phase of *H. pluvialis* cutlers where shade effect is present at 10^{th} day of the second experiments. Before the exponential phase, there were no green light additive effects was observed. That results may indicate that green light supplementation would be effective in dense algae conditions where there is shade effect because of increased algal culture density. Such shade effects in algae culture may provide similar conditions with deep forest ecosystems. Green light might penetrate better into the center of the culture flasks and play an accessory role in light-harvesting to pass the light energy to chlorophyll pigments for photosynthesis. Green light penetration in turbid aquatic systems is higher than red and blue light because of less absorption rate by microalgae. Red and blue light are quickly absorbed by microalgae especially in aquatic ecosystems having higher primary production and they cannot penetrate more than a few cm in culture clones (Schulze et al., 2017).

However, the green light supplementation above the %10 did not result in such additive effects on *H. pluvialis* growth parameters. These results may indicate a dose-dependent green light effect on algae cultures. An excessive amount of green light percentage may suppress photosynthesis and light harvesting metabolisms. The result of the first experiment supports the idea that green light subtraction at % 50 and 100 % enhance *H. pluvialis* growth rate by $1.05x\ 10^5 \pm 6.1x\ 10^3$, $9.27x\ 10^4 \pm 1.32x\ 10^3$, $9.2x10^4 \pm 4x\ 10^3$ observed in G0, G/2 and W treatments at 13^{th} day of experiment respectively. These results are also complementary with previous study addressing the enhancement of red and blue light on microalgae growth (Katsuda et al., 2004; Kim, 2006). Green light ration in supplied light source more than 10% result in suppression in the growth rate and biomass of *H. pluvialis*.

4.2.2 Effect of Green Light Supplementation in the Differential Expression of Selected Candidate Genes in *H. pluvialis*

In order to reveal the transcriptional response of green light supplementation in *H. pluvialis*, we selected four different genes having regulatory roles in PS-I (PsaB), PS-II (PsbS), carbon assimilation (RubisCo) and carotenoid biosynthesis

($PTOX_2$). PsaB, PsbS and RubisCo genes were select to examine the potential transcriptional alterations in carbon assimilation, PS-I and PS-II systems under 10% green light supplementation. We were expecting to observe an upregulation in light-harvesting regulating gene of PsbS in PSII and carbon assimilation gene of RubisCo parallel with growth rate alteration in 10% green light supplementation. $PTOX_2$ genes were selected as a negative control as it is responsible for stress regulation and biosynthesis of carotenoids which is not the case in our experiments. PsaB genes were selected if 10% green light supplementation have an influence on electron transfer system in PSI.

PsaB is a transmembrane protein and located center of electron transfer chain as one of two main subunits of PSI that contains two phylloquinone, several carotenoids, at least eleven different polypeptides and approximately 100 chl molecules. PsaB involves the binding of electron transfer cofactors of P_{700}.

Accordingly, with the change in chl-a amount, a parallel response in the expression of the PsaB gene was expected. It has been reported that the differences in PSI and PSII complexes in plants grown in yellow and red light are parallel to gene transcripts encoding PSI and PSII polypeptides (Glick et al., 1986). In a similar study, it has been shown that red light stimulates the PSI complex, while yellow light stimulates the PSII complex (Deng et al., 1989).

In our study, there were no significant differences in transcriptional expression between the RB, %10 and 20% green light supplementation of G10 and G20 treatments respectively at time t_5. However, there is a significant downregulation in G20 treatment at the end of the experiment (t_{20}). That downregulation in G20 may be related to the suppression of green light effects that result in a decrease in growth rate and chlorophyll concentration and subsequently decrease in PSI activity.

RubisCo is found in all photosynthetic organisms and catalyzes of CO_2 assimilation by means of CBB (Calvin-Benson Bassham) cycle. Although, its central roles in CO_2 assimilation, the affinity of the enzyme is inefficient both in plant and algae species. Reason of the inefficient enzyme activity of RubisCo is linked to its

evolutionary origin in which CO_2 level was higher in the atmosphere than the present atmosphere. The affinity of the enzyme is much lower in aquatic species than terrestrial ones (Parry et al., 2013; Atkinson et al., 2017). Therefore, RubisCo is one of the most abundant enzymes in algae and plant cell (around 50% of soluble protein of the cell content) and required high energy demand process in order to support efficient CO_2 assimilation of algae and plant cells (Moroney and Somanchi, 1999). So, research regarding increase the efficiency of RubisCo proteins is received big attention in the area of plant and algae biotechnology.

Our results show that 10 % green light supplementation increase RubisCo efficiency in *H. pluvialis*. Differentiation transcriptional level of G10 treatment is moderately higher than RG and G20. Also, 20% of green light supplementation of G20 treatment results in a significant downregulation in RubisCo gene. It can be concluded that there would be dose-dependent green light effects on RubisCo gene activity that can be used to stimulate algae growth.

Both eukaryotic algae and plant species would develop regulation mechanisms against photoinhibition damage in the condition of high photosynthetic light intensity which is higher than needed (DalCorso et al., 2008). High light intensity would result in high electron transport in PSII. In such conditions, light antenna complex of the cells, non-photochemical quenching (NPQ) systems, alternative electron transport systems are altered to avoid high light intensity (Rochaix, 2011; Liu et al., 2019). Excess of light energy is primarily making PSII very sensitive as light energy is first captured by light-harvesting systems of PSII. NPQ is very fast responding protection systems that initiated within seconds to dissipate excess light energy from PSII antenna. PsbS proteins are subjected to one of NPQ regulated process called qE (energy-dependent quenching) by several studies (Niyogi et al. 1998; Avenson et al., 2008; Liu et al., 2019). Moreover, PsbS proteins show activity in the regulation of cyclic electron flow to PSI (Kulheim et al., 2002; Vass, 2012). However, the function of PsbS proteins and involved pathways is still fully understood, and the role of PsbS protein regarding control of the excess light stress is controversial (Liu et al., 2019; Roach and Liszkay, 2012).

In the present study, PsbS transcription was found significantly upregulated in 10% green light supplementation and downregulation in 20% green light supplementation of G10 and G20 treatments respectively. The light intensity used in this study was around 25 µmole $m^{-2}s^{-1}$ that high enough to provide light stress for *H. pluvialis*. Therefore, PsbS activity would not be related to the dissipation of excess energy or any light-induced stress regulation process. A significant difference between 10 and 20 % of green light supplementation in PsbS transcriptional response may indicate dose-dependent green light-induced alteration in light-harvesting complex or electron flow in PSI and II. A specific portion of green light intensity among red and blue light sources might manipulate the photochemical process in electron harvesting and cycling electron flow systems in PSI and II.

$PTOX_2$ protein is an oxidoreductase and resides on the thylakoid membrane of algae and higher plants. It involves secondary carotenoid biosynthesis during environmental stresses, especially in high light intensity. $PTOX_2$ gene was used in this study as a negative control to check if there is a light induced stress condition in our experimental setup. Results showed that differential transcriptional expression of $PTOX_2$ gene between treatments is not at a significant level.

Additive green light effects around 10% supplementation and suppressive effects out of this percentage on algae cell growth may also indicate a completive situation to accesses light availability in dense algal conditions. The higher green light proportion in light supply may act as dilution factors for blue and red-light spectrum that may decrease electron capture by PSII antenna. Downregulation expression of PsaB gene in 20% green light supplementation may indicate lower electron flow from PSII to PSI in because of the green light dilution effects. %10 supplementation may be a critical point for algae cells in the dense culture system and may refer to a tradeoff between cost and benefit to continue cell division.

5. CONCLUSIONS AND RECOMMENDATIONS

Recently, algae have started to play an important role in the field of biotechnology. Production of the metabolites of algae in a short time and in large quantities has been contributed many kinds of research in alga biotechnology. The commercially produced *H. pluvialis* continues to increase significantly astaxanthin produced under high-stress conditions. Astaxanthin pigment, this secondary metabolite produced, is a super antioxidant substance. There are lots of research has been done to determine which conditions will be more favourable for the production of astaxanthin by reaching the cell density of *H. pluvialis* in the fastest way and will continue to be made.

Although researches have been done on many different light sources on the production of *H. pluvialis* cultures, there have not been enough studies on the effect of LED lights. Some studies have proven that red light triggers vegetative growth, while blue light triggers astaxanthin production (Xi et al., 2016; Katsuda et al., 2004). However, there is no study on the effect of green light on *H. pluvialis*.

The purpose of this thesis is; In addition to the known red and blue light spectra for more effective growth of *H. pluvialis* cultures, it was investigated how different green light concentrations affect growth and gene expressions. The present study is to first record that show additive green light effects by using both physiological and molecular approaches on algae.

According to the physiological and genetic results of cultures grown by illuminating with different concentrations of green light, a significant difference was observed in G10 treatment of cell number, chl-a amount, density and genetic analysis. Significant upregulation in transcriptional expression of RubisCo and PsbS genes would also indicate the effects of green light on photosynthesis molecular pathways. This study would present pioneering results to use green light in algae biotechnology to improve culture condition and productivity.

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APPENDICES

7. APPENDICES

A.1. List of Chemical.

Chemical	Molecular Formula	Application
Agar (Plant Tissue Grade)		Agar Plates
Boric Acid	H_3BO_3	BG-11 Culture Medium
Calcium Chloride Dihydrate	$Cacl_2$ X 2 H_2O	BG-11 Culture Medium
Chloroform	$CHCl_3$	RNA Extraction
Citric Acid	$C_6H_8O_7$	BG-11 Culture Medium
Cobalt (II) Nitrate Hexahydrate	$Co(NO_3)_2$ X 6 H_2O	BG-11 Culture Medium
Copper (II) Sulfate Pentahydrate	$CuSO_4$ X 5 H_2O	BG-11 Culture Medium
Deoxyribonucleoside Triphosphate (2mm) [Dntps]		Molecular Analyses; PCR
Dimethyl Sulfoxide [DMSO]	C_2H_6OS	Chlorophyll Extraction
Dipotassium Phosphate Trihydrate	K_2HPO_4 X 3 H_2O	BG-11 Culture Medium
Ethanol (99.8%)	C_2H_6O	Molecular Analyses; RNA Extraction
Ethidium Bromide (1%) [Etbr]	$C_{21}H_{20}BrN_3$	Molecular Analyses; Gel Electrophoresis
Ethylenediaminetetraacetic Acid [EDTA; Titriplex II]	$C_{10}H_{16}N_2O_8$	BG-11 Culture Medium
Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate [NA2EDTA; Titriplex III]	$C_{10}H_{14}N_2Na_2O_8$ X 2 H_2O	BG-11 Culture Medium
Ferric Ammonium Citrate	$(NH_4)_5[Fe(C_6H_4O_7)_2]$	BG-11 Culture Medium
DNA Ladder		Molecular Analyses; Gel Electrophoresis
Loading Dye (6x)		Molecular Analyses; Gel Electrophoresis
Iron (II) Sulfate Heptahydrate	$FeSO_4$ X 7 H_2O	BG-11 Culture Medium
Isopropyl Alcohol	C_3H_8O	Molecular Analyses; RNA Extraction
Magnesium Sulfate Heptahydrate	$MgSO_4$ X 7 H_2O	BG-11 Culture Medium
Manganese (II) Chloride Tetrahydrate	$MnCl_2$ X 4 H_2O	BG-11 Culture Medium
Nuclease-Free Water	H_2O	Molecular Analyses; PCR; Sequencing
Potassium Chloride	KCl	Molecular Analyses
Sodium Chloride	NaCl	Molecular Analyses
Sodium Hydrogen Phosphate	Na_2HPO_4	Molecular Analyses
Sodium Molybdate Dihydrate	Na_2MoO_4 X 2 H_2O	BG-11 Culture Medium
Sodium Nitrate	$NaNO_3$	BG-11 Culture Medium
Sodium Thiosulfate Pentahydrate	$Na_2S_2O_3$ X 5 H_2O	Agar Plates
Thiamine Hydrochloride (Vitamin B1) [Thiamine-Hcl]	$C_{12}H_{18}Cl_2N_4OS$	BG-11 Culture Medium
Tris (Hydroxymethyl) Aminomethane [Tris]	$C_4H_{11}NO_3$	Molecular Analyses; RNA Extraction; Gel Electrophoresis
Zinc Sulfate Heptahydrate	$ZnSO_4$ X 7 H_2O	BG-11 Culture Medium

A.2. Equipment used in this study.

EQUIPMENTS	MODEL
+4 °C refrigators	Arçelik
-20°C deepfreeze	Arçelik
-80°C deepfreeze	Infrico medcare-ULF400
Air compressor	Atlas- SC 1025
Autoclave	Selecta MED20
Biosafety Cabinet	Miprolab-Class2A2
Centrifuge	Thermo Scientific MicroCL 17R
Distiller machine	ELGA VEOLIA
Electrophoresis system	Thermo Scientific EC 300XL
Heatblock	BIOER CHB-202
Homogenizer	BeadBug D1030-E
Incubator	Miprolab-MSI120
Magnetic stirer	VELP Scientifica
Micropipetts	Eppendorf Research plus
Microscope	Motic BA310E
Microwave oven	Arçelik
PCR	BIOER TC-E-96G
PH meter	HANNA
Real Time PCR	Sub-Module: BioRad C1000 Touch Top Module: BioRad CFX96
Spectrophotometer	VWR UV-6300PC
Spin	Thermo Scientific MySPIN6
Vortex	VELP Scientifica

A.3. Cell count of both experiments.

Treatment	7th days	11th days	15th days	20th days
Experiment 1				
W(white light)	42.222 mL^{-1}	77.333 mL^{-1}	103.333 mL^{-1}	-
G/2	45.000 mL^{-1}	75.833 mL^{-1}	94.444 mL^{-1}	-
G0	55.833 mL^{-1}	98.500 mL^{-1}	98.111 mL^{-1}	-
Experiment 2				
RB (red and blue light)	57.222 mL^{-1}	107.222 mL^{-1}	134.444 mL^{-1}	212.143 mL^{-1}
G10	59.444 mL^{-1}	116.111 mL^{-1}	172.222 mL^{-1}	245.540 mL^{-1}
G20	55.556 mL^{-1}	115.000 mL^{-1}	162.778 mL^{-1}	223.333 mL^{-1}

A.4. Amount of Chl-a in both experiments.

Treatment	7th days	11th days	15th days	20th days
Experiment 1				
W(white light)	2.75 µg mL^{-1}	4.69 µg mL^{-1}	7.77 µg mL^{-1}	-
G/2	2.17 µg mL^{-1}	4.38 µg mL^{-1}	7.77 µg mL^{-1}	-
G0	2.75 µg mL^{-1}	5.33 µg mL^{-1}	10.12 µg mL^{-1}	-
Experiment 2	**8th days**	**12th days**	**16th days**	**20th days**
RB (red and blue light)	3.99 µg mL^{-1}	7.47 µg mL^{-1}	13.18 µg mL^{-1}	15.25 µg mL^{-1}
G10	4.01 µg mL^{-1}	7.61 µg mL^{-1}	14.34 µg mL^{-1}	15.92 µg mL^{-1}
G20	3.89 µg mL^{-1}	8.14 µg mL^{-1}	12.98 µg mL^{-1}	15.18 µg mL^{-1}

A.5. OD values of both experiments.

Treatment	2th days	3th days	4th days	5th days
Experiment 1				
W(white light)	0.03	0.05	0.06	0.07
G/2	0.03	0.03	0.06	0.07
G0	0.04	0.07	0.07	0.08
Experiment 2	**8th days**	**12th days**	**16th days**	**20th days**
RB (red and blue light)	0.09	0.16	0.22	0.31
G10	0.09	0.18	0.24	0.34
G20	0.09	0.15	0.22	0.30

8. CURRICULUM VITAE

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