DOKUZ EYLÜL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

OPTIMIZATION OF ECONOMICAL MICROALGAL LIPID PRODUCTION AND INVESTIGATION OF BIOFUEL POTENTIAL

by

Zülfiye VELİOĞLU TOSUNER

September, 2021

İZMİR

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We have read the thesis entitled **"OPTIMIZATION OF ECONOMICAL MICROALGAL LIPID PRODUCTION AND INVESTIGATION OF BIOFUEL POTENTIAL"** completed by **ZÜLFİYE VELİOĞLU TOSUNER** under supervision of **PROF.DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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OPTIMIZATION OF ECONOMICAL MICROALGAL LIPID PRODUCTION AND INVESTIGATION OF BIOFUEL POTENTIAL

ABSTRACT

Fossil fuels are approaching the point of depletion. Therefore, the interest in renewable energy sources is increasing. Biofuels produced from various biological sources are popular energy alternatives. Biodiesel production from microalgae has been an interesting field of study.

In this study, lipid production was carried out under different nutritional and environmental conditions using a green algae, *Haematococcus pluvialis*, and a blue-green algae, *Spirulina platensis*.

H. pluvialis had the highest lipid production in mixotrophic medium containing two point five millimolar crude glycerol and twelve times higher production was achieved than in photoautotrophic medium. *S. platensis* produced the highest lipid production in medium containing ten millimolar crude glycerol, with approximately nineteen percent more production than in photoautotrophic medium. The fatty acid profile of the produced lipid was determined by gas chromatography and its biodiesel potential was investigated. The lipid produced is in compliance with European and American standards in terms of parameters such as iodine value, cetane number and oxidation stability, which are indicators of fuel quality.

Evaluation of waste in obtaining value-added products emphasizes the economic and ecological aspects of the study. The residue formed at the end of lipid extraction from the cell has the potential to be utilized in different areas due to its protein and carbohydrate content. This approach also supports zero waste studies. Additionally, the potential of two different microalgae species, which have not been adequately studied in terms of biodiesel production, has been revealed in terms of quality biodiesel production.

Keywords: *Haematococcus pluvialis*, *Spirulina platensis*, crude glycerol, technical glycerol, whey, mixotrophic culture, heterotrophic culture, lipid production, biodiesel

EKONOMİK MİKROALGAL LİPİD ÜRETİMİNİN OPTİMİZASYONU VE BİYOYAKIT POTANSİYELİNİN İNCELENMESİ

ÖZ

Fosil yakıtlar tükenme noktasına yaklaşmaktadır. Bu sebeple yenilenebilir enerji kaynaklarına olan ilgi artmaktadır. Çeşitli biyolojik kaynaklardan üretilen biyoyakıtlar popüler enerji alternatifleridir. Mikroalglerden biyodizel üretimi ilgi çekici bir çalışma alanıdır.

Bu çalışmada bir yeşil alg olan *Haematococcus pluvialis* ve bir mavi-yeşil alg olan *Spirulina platensis* kullanılarak farklı besinsel ve çevresel koşullarda lipid üretimi gerçekleştirilmiştir.

H. pluvialis, en yüksek lipid üretimini iki buçuk milimolar ham gliserol içeren miksotrofik ortamda ve fototrofik ortama göre on iki kat daha yüksek gerçekleştirmiştir. *S. platensis*, en yüksek lipid üretimini on milimolar ham gliserol içeren ortamda ve fototrofik ortama göre yaklaşık yüzde on dokuz daha fazla gerçekleştirmiştir. Üretilen lipidin yağ asidi profili gaz kromatografisi ile belirlenmiş ve biyodizel potansiyeli araştırılmıştır. Yakıt kalitesinin göstergesi olan iyot değeri, setan sayısı ve oksidasyon stabilitesi gibi parametreler açısından üretilen lipid Avrupa ve Amerika standartlarına uygundur.

Atıkların katma değerli ürün eldesinde değerlendirilmesi çalışmanın ekonomik ve ekolojik yönünü vurgular. Hücreden lipid ekstraksiyonu sonunda oluşan kalıntının da protein ve karbohidrat içeriği sebebiyle farklı alanlarda değerlendirilebilme potansiyeli vardır. Bu yaklaşım sıfır atık çalışmalarını da destekler niteliktedir. Ayrıca biyodizel üretimi açısından yeterli derecede incelenmemiş olan iki farklı mikroalg türünün kaliteli biyodizel üretimi açısından potansiyelinin ortaya çıkarılması sağlanmıştır.

Anahtar kelimeler: *Haematococcus pluvialis*, *Spirulina platensis*, ham gliserol, teknik gliserol, peynir altı suyu, miksotrofik kültür, heterotrofik kültür, lipid üretimi, biyodizel

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CHAPTER ONE
INTRODUCTION

Energy is the one of main factors for the sustainable life worldwide. In recent years the interest in the search for substitute energy sources has increased due to the need for energy has increased while the fossil fuel reserves have reached the point of exhaustion (Machado & Atsumi, 2012). The fact that the most important cause of greenhouse gas accumulation and global climate change is fossil fuels supports this interest. Total primary energy consumption in 2019 was around 583.9 exajoules. While 33.05% of this energy need is met from oil, 24.23% from natural gas, 27.04% from coal, 4.28% from nuclear energy, 6.45% from hydroelectric energy, only 4.96% of them were met from renewable energy sources (BP, 2020). Turkey, while achieving 81.51% of the total energy needs (6.46 exajoules) from fossil sources (oil, coal and natural gas) in 2019 was provided with only 6.31% of renewable energy sources (BP, 2020). In today's world where global climate change is approaching alarming rates irreversible, urgent and effective measures to reduce CO_2 emissions are required (Anca-Couce, Hochenauer & Scharler, 2021). Renewable energy such as biofuels takes the important part of the reduction of greenhouse gases strategies.

Renewable energy sources are remarkable alternatives of fossile fuels and studies and legal regulations for the production and use of these fuels in the world and Turkey has gained importance in recent years. Among the objectives of the 2011-2023 Climate Change National Action Plan, organized by the Ministry of Environment and Urbanization, is directly and indirectly to promote the production and use of renewable energy sources (İklim Değişikliği Ulusal Eylem Planı, 2020). While the production of biofuels has increased by 5.1% worldwide, large-scale production is not yet carried out in Turkey (BP, 2020).

1.1 Biofuels

Energy obtained from renewable sources is necessary for life to continue without disturbing the ecological balance. Biofuels, produced from different biological sources, stand out with their high energy contents and low CO_2 emission profiles (El-

Shimi, Attia, El-Sheltawy & El-Diwani, 2013). Biofuels are divided into four sorts according to the origin from which they are produced. First generation biofuel sources are designated as “energy plants” like corn, sweet sorghum, sugarcane etc. There are plenty of successful studies and applications for biodiesel production from these plants. In recent years, with the raise of the inhabitants in different parts of the world the problem of hunger is gradually increasing, but the land area in agricultural productivity is rapidly decreasing (Rezania et al., 2020). In these conditions, the use of agricultural lands for food production is a priority. Second generation sources for biofuel production are waste oil, several agro-industrial wastes, forest residues, nonedible food, etc. The main problem about the second generation sources is insufficient amount to respond the energy demand. Microbial biomasses, such as microalgae, are the third generation sources with advantages of high productivity and low area need according to energy plants. The fourth generation sources are genetically modified microalgae which have conflict about the possible negative effects on environment and human health (Abdullah et al., 2019). Biofuels are also categorized into four classes such as biogas, biodiesel, biohydrogen and bioethanol (Rezania et al., 2020). Biodiesel is a natura friend alternative to conventional diesel with easy and economic production process (Gebremariam & Marchetti, 2018).

Bioethanol (C_2H_5OH or EtOH) is an eco-friendly and mostly preferred biofuel and it is produced by fermentation of sugar. Ethanol, which is produced from sugar cane in Brazil and corn sugar in America, is two of the most widely used in the world (Zabed, Sahu, Suely, Boyce & Faruq, 2017). It is a fuel that can be produced as the first generation when vegetable sugar is used, as the second generation when lignocellulosic waste is used, and as the third generation when microbial sugar is used. It stands out with its higher combustion efficiency, lower solid particle and NOx emission compared to gasoline. In addition, thanks to its high oxygen content, it is a much cleaner fuel than gasoline (Aditiya, Mahlia, Chong, Nur & Sebayang, 2016). It can be also mixed with gasoline or used as immaculate alcohol in engines (Chen & Qiu, 2010). Difficulties in providing raw materials and production costs are the biggest problems faced in the widespread use of bioethanol.

Biobuthanol is also another attractive liquid energy source. It is a product of acetone-buthanol-ethanol production process (Amiri & Karimi, 2019). It is usually produced by anaerobic bacteria such as Clostridia in a growth medium with a high sugar content. It is seen as an important alternative to gasoline and ethanol. Two most important problems encountered in biobutanol production are the need for strict anaerobic conditions and the inhibitory effect of the final product after a certain concentration (Kumar & Gayen, 2011).

Biohydrogen is one of sustainable, renewable fuel. Use of hydrogen generates only water and energy (Usman, Banu, Gunasekaran & Kumar, 2019). It is a potent alternative fuel because it provides clean energy and transforms into energy with high efficiency (Elsharnouby, Hafez, Nakhla & El Naggar, 2013). Biohydrogen could be generated from a broad range of carbohydrates such as glucose, sucroce, agricultural wastes, lignocellulosic products etc. It is an effective strategy to use waste materials as substrates in order to reduce the high production cost, which is one of the most important problems encountered in biohydrogen production (Cheng et al., 2011). A large type of bacteria manages to produce biohydrogen such as Clostridia, Escherichia, Enterobactericeae, cynobacteria (Show, Lee, Tay, Lin & Chang, 2012). The difficulty of storage and transfer conditions is one of the most important obstacles to the widespread use of hydrogen.

Biogas mainly involves methane (50-75%), CO_2 (25-50%) and water vapour (Plugge, 2017). It is released as a result of the digestion of various organic materials by anaerobic microorganisms. The energy potential depends on the methane content. Many diverse kinds of waste such as agricultural and industrial waste, municipal waste and sewage can be used as an organic source in the generation of biogas (Scarlat, Dallemand & Fahl, 2018). Although it is easy to use and common in Europe, there are various problems during the production phase. There are important variables to consider, such as the challenges of the anaerobic environment, the pretreatment requirement of the organic material, and the needs of the bacterial co-culture used.

Although different renewable energy sources like solar, wind, wave, hydraulic and geothermal are used in Turkey, the use of biomass energy remains at low levels (Kaya,

Şenel & Koç, 2018). Turkey has a potential of approximately 117 billion tons of biomass/year, with this potential, 371.2 TWh/year electrical energy can be produced. Most of this potential consists of annual plants, forest wastes and perennial plants. There are private sector or municipal facilities for the generation of biogas, bioethanol and biodiesel in different regions of Turkey. These facilities are controlled by the Ministry of Energy and Natural Resources. The most striking detail is that the resources used for bioenergy production are first and second generation sources such as animal/plant origin materials, forest wastes and municipal wastes (Türkiye Biyokütle Enerji Atlası, 2021). It is seen that the effect and importance of microbial biomass in bioenergy production is not yet fully realised.

1.2 Biodiesel

Biodiesel is a non-toxic, biodegradable, easily flammable diesel alternative liquid renewable fuel. It includes long chain fatty acids' mono alkyl esters (Dahiya, 2014). Edible and non-edible oils are potential biodiesel feedstock such as soybean, canola, sunflower, mustard, rapeseed oils, animal fats, used edible oils, fish oil and tallow oil (Rezania et al., 2020) (Figure 1.1). Biodiesel does not contain petroleum but can be blended with petroleum diesel, and these mixtures can be used in compression ignition (diesel) engines with minor or no alteration. The blending of petroleum diesel with biodiesel causes slight power loss, while it provides reducing CO, hydrocarbons and particulate matter emissions (Malode, Prabhu, Mascarenhas, Shetti & Aminabhavi, 2021). Additionally, to performing a clean combustion, biodiesel is an important alternative biofuel because it is easy to use, non-toxic, biodegradable, renewable and free from sulfur and aromatics that admits it to have minimize toxicity and pollutant emissions (Dahiya, 2014; Gebremariam & Marchetti, 2018; Rezania et al., 2020). In addition to this biodiesel generates fewer carbon monoxide, smoke, hydrocarbons and particulate matters. Due to biodiesel has higher free oxygen than petroleum diesel, it shows complete combustion and decreased emission (Gebremariam & Marchetti, 2018).

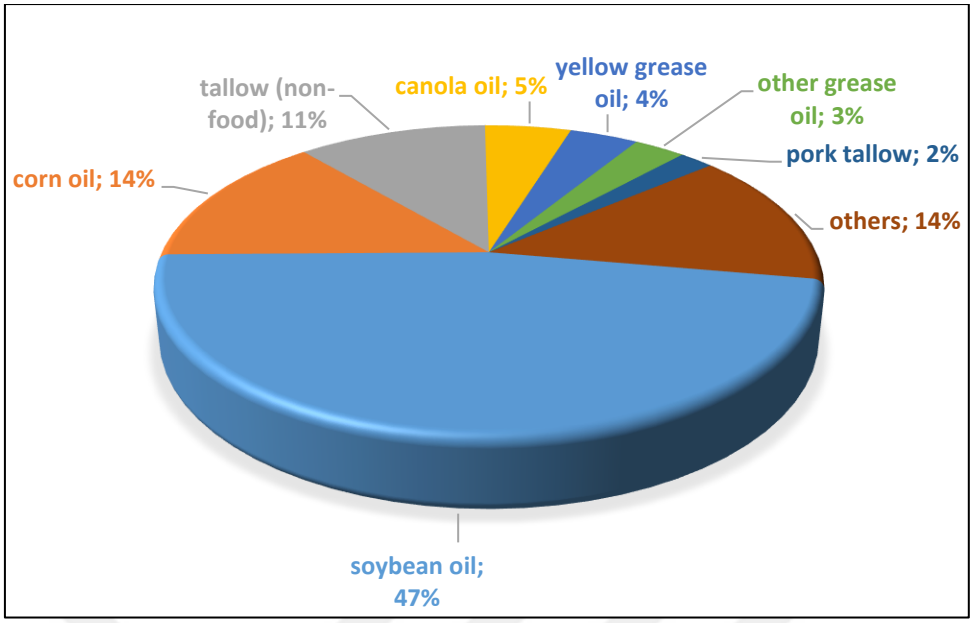

Figure 1.1 Various raw materials used in biodiesel production (Dahiya, 2014)

There are two different biodiesel production methods called direct (*in-situ*) or indirect transesterification (Karpagam, Jawaharraj & Gnanam, 2021). For the indirect transesterification process first, the lipid has to be extracted from biomass, FAME is then produced. In the direct (*in-situ*) transesterification process, FAME production is carried out in one step without the need for lipid extraction (Figure 1.2). In transesterification reaction glycerol is separated from oil and three moles methyl esters and one mole glycerol are formed at the end of the mechanism (Figure 1.3). Biodiesel is produced in an amount that can absorb 5.54 units of energy per unit of energy used in this reaction (Dahiya, 2014).

The most effective parameters on transesterification process yield are alcohol/ oil ratio, temperature, catalyst class, moisture and reaction time (Karpagam et al., 2021). Generally, acid (HCl, H_2SO_4), base (NaOH, KOH) or enzymes are used as catalyst. No soap formation occurs with acid catalyst and acid catalysis is suitable for direct transesterification. However, the risk of corrosion in the reactor and the low rate of reaction are the disadvantages of the acid catalyst. Due to high production yield, short reaction time at low pressure and temperature, base catalysis is the most preferred one. The most important handicap is the formation of soap as a result of its reaction with oils containing high free fatty acid. In the enzyme catalysis systems lipase is used

because of its catalization efficiency on trigliseride and also free fatty acids. There are important advantages such as high conversion yield, high biodiesel purity, low energy need. The factors preventing its widespread use are its high cost and the possibility of inhibition of the enzyme.

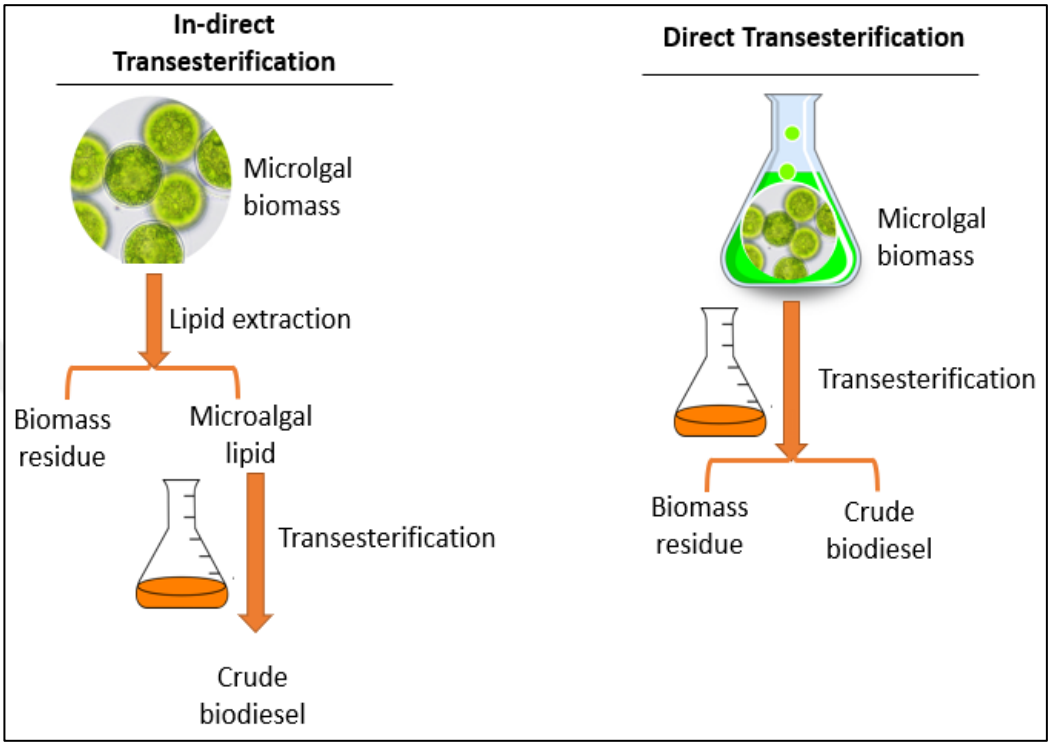

Figure 1.2 Biodiesel production by transesterification (Karpagam et al., 2021)

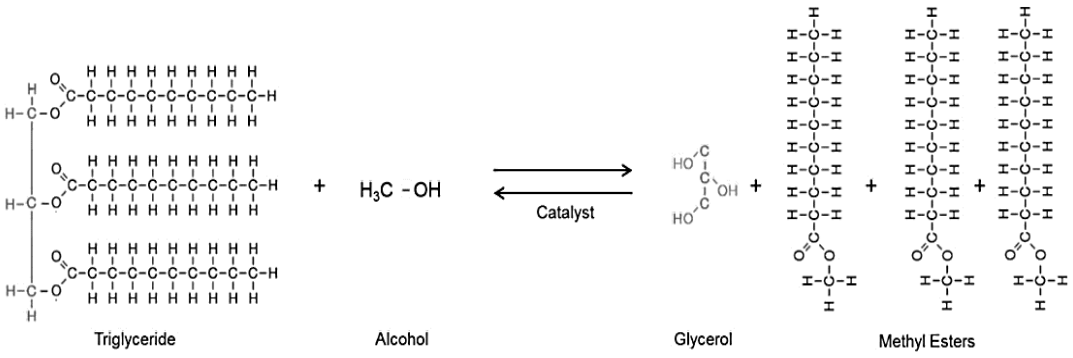

Figure 1.3 Transesterification reaction (Pollitt, Chhan, Rais, Pan & Wallace, 2019)

1.2.1 Qualitative Biodiesel Standards

Fuel grade biodiesel must be produced according to some standards to ensure correct and high effciency (Rós et al., 2013). The cetane (hexane) number is a measure of the ignition capacity of diesel as compression ignition and it is an important definition of diesel quality. Biodiesel generally has a higher cetane number (42-44) compared to diesel fuel (Lower limit according to European standards (UNE-EN 14214) 51), which makes biodiesel a more combustible fuel. The biodiesel's flashing point of is higher than diesel (> 110ºC). This feature provides a safer fuel for the use, transportation and storage of biodiesel. Iodine value, another quality criterion of biodiesel, is the amount of iodine in grams necessary for saturation of double bonds in 100 grams of oil and the acceptance range according to European standards (UNE-EN 14214) is 57-100 g I_2/ 100 g. Unsaturation causes sediment and storage stability problems. High iodine count is not recommended due to excessive carbon residue formation. Correctly processed biodiesel has the same range of viscosity as conventional diesel; however, the viscosity of the unprocessed vegetable oil is much higher than that of diesel (Dahiya, 2014). According to European standards (UNE-EN 14214), the acceptance range of a quality biodiesel at 40°C is 3.5-5 mm^2/sec. Sulfur in petroleum diesel provides lubricity, but due to its harmful effects on the environment, the use of sulfur-free diesel is required by law (Rós et al., 2013). However, biodiesel has high lubricity in its natural form and it is mixed into petroleum diesel in order to provide lubricity to the sulfur-free diesel. In addition, the cold flow properties (cloud point, slip point) of biodiesel are better than petroleum diesel, which makes it possible to use in different climatic conditions.

In order to produce quality biodiesel, suitable quality raw materials are needed. The water content of the lipid to be used as a raw material should not be higher than 1%, otherwise the transesterification process will not be completed and soap formation will occur. Besides that, rough materials involve great levels of free fatty acids will consume large amounts of base catalysts and form soap. For this reason, the raw material should not contain more than 1% free fatty acid. Furthermore, low particulate matter, phospholipid and sulfur content will increase the biodiesel quality to be obtained.

1.2.2 Suitable Fatty Acid Profile for Biodiesel Production

Fatty acid profile of produced lipid is the main factor that affects the quality of biodiesel. The main methyl esters for a quality biodiesel production are palmitate (C16:0), stearate (C18:0), oleate (C18:1), linoleate (C18:2), and linolenate (C18:3) (Shin et al., 2018). The optimum proportion of fatty acid for biodiesel production is 5:4:1 of C16:1, C18:1, and C14:0, respectively (Schenk et al. 2008). The cetane number (CN), iodine value (IV), saponification value (SV), degree of unsaturation (DU), oxidative stability (OS) and long chain saturated factor (LCSF) values calculated using the fatty acid profile are used to determine the quality of the produced biodiesel (Rós et al., 2013).

CN is indicator of the ignition capacity. High cetane number means good start characteristics in low temperature and decreases the creatin of white smoke (Ramos, Fernández, Casas, Rodríguez & Pérez, 2009). Higher content of long and more saturated fatty acid causes higher CN. As DU increases, the number of cetane also increases. Biodiesel generally has a higher CN than diesel fuel and this makes biodiesel a more combustible fuel.

IV is the amount of iodine in grams needed to saturate double bonds in 100 grams of oil (Sivaramakrishnan & Incharoensakdi, 2018). Higher DU leads to higher IV values. IV is related to deposits and storage stability problems. Besides, OS is the main property that affects the safety of use. The content of polyunsaturated fatty acid affects the OS value (Ramos et al., 2009). Some committees were studied on standardization of requirements and test methods for common type biodiesel production from FAME. European Standard (UNE-EN 14214) and American Society for Testing and Materials (ASTM 675) standards are most common tests. These standards focus on IV, CN and OS values.

The fact that biodiesel produced from microalgal and/ or bacterial oils indicate that this fuel is more promising fuel for the future. Microalgae are important alternatives for biodiesel production with high growth rates, high biomass efficiencies and 15-300 times more oil contents compared to grain products. Besides, it is an important

advantage that fuel production from microalgal oils does not compete with existing food and water resources, and industrial, urban or agricultural wastes can be used in production environments.

1.3 Microalgae

Microalgae are photosynthetic organisms which produce biomass, biomolecules and O_2 by using solar energy for energy need and CO_2 for carbon need (Patel, Gami, Patel & Patel, 2016). Some of them are unicellular and the rest are simple multicellular prokaryotic or eukaryotic microorganisms. Blue-green algae, also called cyanobacteria, are prokaryotic microalgae. Microalgae are primary producers and approximately 50% of carbon is fixed by microalgae to generate O_2 (Katiyar et al., 2017). Many microalgae type have movement ability through flagella. In addition to aquatic environments such as seas, lakes, artificial pools, and aquariums, they can also survive in moist soils. They have unicellular, colony or filamentous morphological properties in their natural environment.

Depending on the species and production conditions microalgae contain protein (30-40%), lipid (10-20%) and carbohydrate (5-15%) (Table 1.1). Due to rich biochemical content, they are used as functional foods (Katiyar et al., 2017). In addition, as a result of the various metabolites such as vitamins, carotenoids and pigments, use of microalgae in food, pharmacy and medicine is becoming widespread. Moreover, the biosorption and adsorption capabilities of microalgae are used in the treatment of some wastewater.

Microalgea survive by photosynthesis which is a complex and vital process. The solar energy is catched by pigments and used to convert the water and CO_2 into glucose and O_2. In this process, so many reaction and enzymes are used. Chlorophyll is the main pigment molecule which is responsible for this mechanism. Although there are different chlorophyll molecules, the most commen ones in microalgae are chlorophyll a and b.

Table 1.1 Protein, carbohydrate and lipid contents by weight of various microalgae species (Katiyar et al., 2017)

Microalgae species	**Protein (%)**	**Carbohydrate (%)**	**Lipid (%)**
Scenedesmus obliquus	50–56	10–17	12–14
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Porphyridium cruentum	28–39	40–57	9–14
Spirulina platensis	46–63	8–14	4–9
Spirulina maxima	60–71	13–16	6–7
Haematococcus pluvialis	29–45	15–17	20-45

1.3.1 Microalgae in Biofuel Production

Nowadays, microalgae are considered as important sources in various biofuels production due to their high growth rates and oil content, helping to reduce air pollution and O_2 production by using CO_2 and not requiring as large areas as agricultural land for their production (Figure 1.4) (Simasatitkul, Gani & Arpornwichanop, 2012).

Microalgae do not need very complex nutrient media for growth, and their ability to adapt to different environmental conditions is quite high (Katiyar et al., 2017). As mentioned in the previous section, besides having high lipid content, the generation times vary between 1-3 days provides an important advantage in terms of fast production. This enables more harvests during the year compared to traditional energy crops. In addition, it is known that various bioreactors, photobioreactors or pool systems used in the production of microalgae require 49–132 times smaller areas compared to agricultural lands.

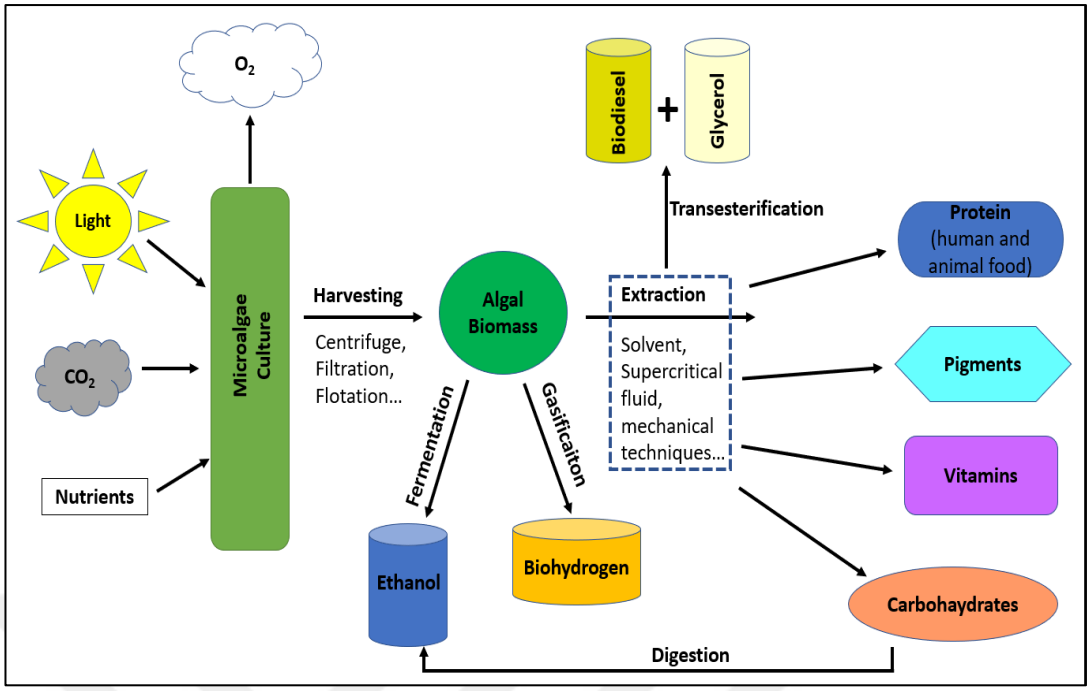

Figure 1.4 Various biofuels and biomaterials that can be produced from microalgal biomass (Katiyar et al., 2017)

While selecting the microalgae species to be used in biofuel production, high biomass production and lipid yields are the main criterions. Moreover, factors such as low production cost, synthesis of valuable by-products, high growth rate, and easy harvesting of cells from the growth medium are important parameters affecting the selection (Brennan & Owende, 2010; Mutanda, et al., 2011).

For qualitative biodiesel production, qualitative and meeting certain criteria lipid is required. In the optimum growth conditions, microalgae generally produce lipid in limitid amounts (Goh et al., 2019). When the microalgae is in the environmental stress such as nutrient starvation, high light intensity, pH and temperature changes, etc. it gathers lipid as fatty acid methyl ester for the carbon and energy storage of cell. Nevertheless, in this stress conditions for lipid production, it generally induces protein biodegradation which may inhibit cell growth (Shin et al., 2018). For these reasons microalgae production conditions are the main criteria for biodiesel production from microalgae.

1.3.2 Lipid Production from Microalgae

The productivity and quality of produced lipid by microalgae depend on microalgae type, cultivation mode, components of production media, light intensity photosynthetic activity (Brennan & Owende, 2010). Microalgae cell contains different fatty acid types such as free fatty acids (FFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acids (PUFA), and saturated fatty acid (SUFA) (Shin et al., 2018). Cell and organelle membranes contain polar lipids even though neutral lipids are used as energy reservoir (Zhu, Li & Hiltunen, 2016). Microalgae store neutral lipids in the form of triacylglycerol (TAG). Microalgae use carbohydrates produced by photosynthesis in the production of acetyl-CoA with more than one different pathway. Acetyl-CoA is the precursor molecule in the fatty acid biosynthesis (Figure 1.5).

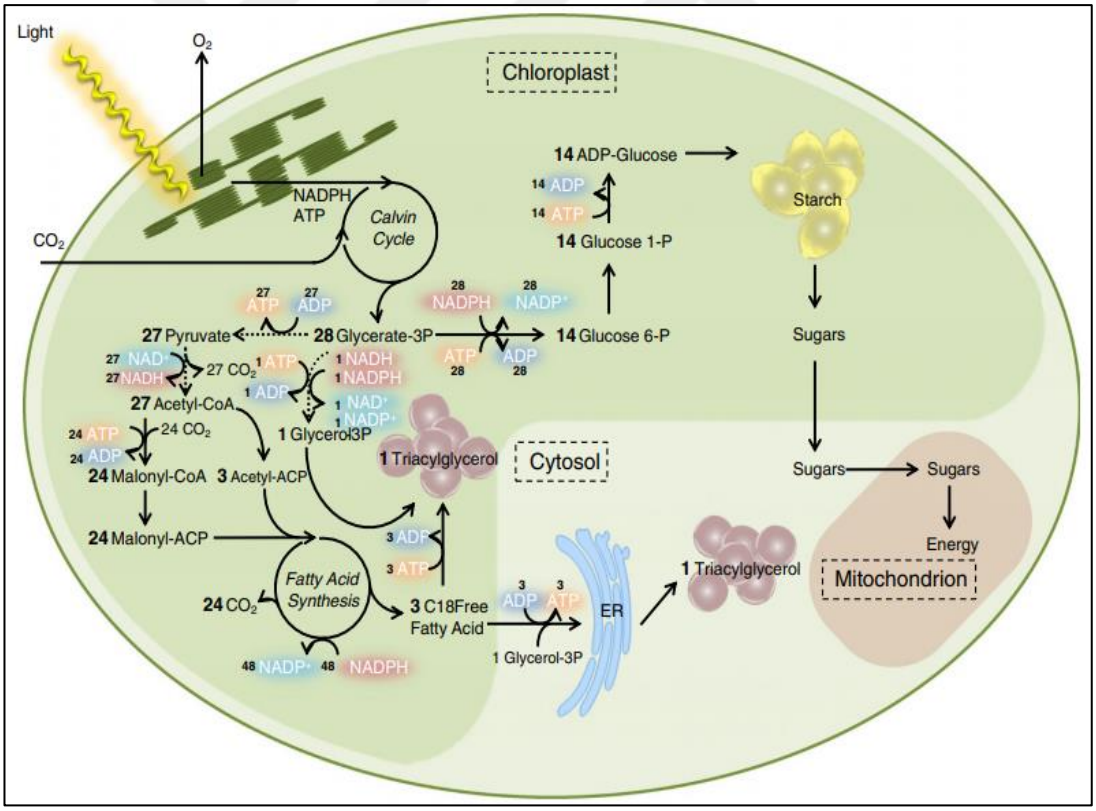

Figure 1.5 Stylized TAG and starch metabolism in green microalgae (De Jaeger et al., 2014)

Green microalgae cells catch the light by specialized protein complexes which expression is dependent on light intensity (Schenk et al., 2008). The activation energy

starts photosynthetic reactions by transferring to photosynthetic reaction centers, photosystem I and II (PSI and PSII). In the first step water transforms to protons, electrons and oxygen with the energy in PSII. The electrons move along the photosynthetic electron transport chain and reach NADPH. Concurrently, protons are delivered into the thylakoid lumen by PSII. This causes a proton gradient and ATP production. The produced NADPH and ATP are spent in biochemical pathways like Calvin cycle to generate biomolecules such as starch, lipid etc.

Fatty acids are main parts of several cellular lipids containing TAG (Katiyar et al., 2017). Microalgae firstly synthesis fatty acids and then produce membrane lipids based on glycerol by esterification reaction, in the optimum growth conditions. In the unexpected environmental stress conditions, they transform lipid biosynthesis pathway to accumulate neutral lipids as TAG forms thus they generate energy storage (Roleda et al., 2013). A multifunctional enzyme complex acetyl-CoA carboxylase (ACCase) has an important role to generate malonyl-CoA from acetyl-CoA and bicarbonate in the fatty acid synthesis pathway (Katiyar et al., 2017). Lastly, produced fatty acids enter the endoplasmic reticulum for glycolipid synthesis. TAG synthesis in microalgae changes depending on alteration of ACCase activity. In the next steps, TAGs are produced by consecutively acylation of three acetyl-CoA with glycerol-3-phosphate. Depending on the type of microalgae, the content of the TAGs may alter. Microalgae, unlike other energy plants, can reach high TAG amount due to high surface area/ volume ratio (De Jaeger et al., 2014). This ratio may increase nutrient uptake and the production of TAG required for biodiesel production. In addition, this ratio allows microalgae to endure environmental stresses, making them a more important alternative for energy production from other earthly plants. Cyanobacteria are able to synthesize simpler fatty acids than eukaryotic microalgae, and this fatty acid profile is also very similar to seed oils, which have been successfully used as lipid reserves for biodiesel synthesis.

1.3.3 Cultivation Types for Microalgae Production

Mainly microalgae are cultivated in photoautotrophic cultures. In the photoautotrophic cultures, microalgae use light as energy source and CO_2 as carbon source in the photosynthetic metabolic pathway (Gonzalez-Toril & Pereto´, 2011). The produced proton motive force in this pathway is used in the synthesis of ATP and reducing power. Photoautotrophic cultivation is an easy-to-use method but it is challenging to arrive high microalgae biomass due to the limited light diffusion and carbon fixation in the culture medium (Hamza, Hamouda, Husein & Abd-Elwahid, 2013). Determination of the optimum light intensity is an important step for the photoautotrophic cultivation of microalgae. At low light intensity, light transmission to the inner parts of the culture medium will be prevented due to cell density. In contrast, high light intensity can cause photoinhibition. Photoinhibition is defined as the damage of photosynthetic apparatus due to various environmental factors such as high light intensity (Raven, 2011). Due to the seasonal changes during the year in photoautotrophic microalgae the production in a commercial-size open system, biomass yield decreases with the decrease of photon flow from sunlight (Paranjape, Leite & Hallenbeck, 2016).

As a solution to this problem in photoautotrophic cultures, heterotrophic and mixotrophic cultivation types are enhanced (Table 1.2). In heterotrophic cultures microalgae are grown in the medium that contains an organic carbon and energy source but there is no light (Hamza et al., 2013). This cultivation type provides high biomass production. The other cultivation type, mixotrophic culture, is a combination of photoautotrophic and heterotrophic cultures. Mixotrophic cultures had light as energy source and an external organic carbon source. It has been determined that microalgal cell concentration, and lipid production efficiency increase and photoinhibition effect decreases in mixotrophic cultures (Chojnacka & Noworyta, 2004).

The main advantages of heterotrophic and mixotrophic cultures are rapid growth rates and increasing biomass quality (Li et al., 2020). Unfortunately, certain microalgae cannot exist in the heterotrophic cultures. This can be explained in three possible reasons: a) lack of transmembrane systems, b) lack of some key enzymes, c)

poor respiration ability. Therefore, many microalgae species can be more easily adapted to mixotrophic culture. The existence of an organic carbon source in the growth medium may decrease the CO_2 absorption and photosynthetic carbon fixation efficiency of microalgae (Li et al., 2020). The common organic carbon source, glucose, is generally metabolized via pentose phosphate pathway in heterotrophic metabolism and via glycolytic pathway in autotrophic metabolism (Yang, Hua & Shimizu, 2000, Velioğlu Tosuner and Öztürk Ürek, 2020). Due to the fact that the complicated assimilation mechanisms can vary from species to species, in heterotrophic and mixotrophic cultures how microalgae can adjust their metabolism is not clear yet (Perez-Garcia, Escalante, De-Bashan & Bashan, 2011).

Table 1.2 Comparison of photoautotrophic, mixotrophic and heterotrophic cultivation types (Zhan, Rong & Wang, 2017)

Cultivation type	Carbon source	Energy source	Advantages	Disadvantages
Photoautotrophic	Inorganic carbon (CO_2)	Light	• Low cost • Simple application • High pigment production	• Low biomass production • Reliance of climate for large scale
Mixotrophic	Inorganic carbon (CO_2) and organic carbon (glucose, acetate, glycerol etc.)	Light and organic carbon	• High grow rate and biomass production • Maintenance of pigment production • Low CO_2 release	• Higher cost • High contamination risk • Decreased energy conversion capability
Heterotrophic	Organic carbon	Organic carbon	• High grow rate and biomass production • Easy reactor design	• Higher cost • High contamination risk • Low pigment production • CO_2 release

In the study of Chojnacka and Noworyta (2004), the growth properties of *Spirulina* sp. were investigated in heterotrophic, mixotrophic and phototrophic cultures. They indicate that the cyanobacteria were adapted to three of the cultivation types and the maximum growth occurs in glucose concentrations higher than 0.5 g/ L in the presence of light higher than 30 W/ m^2. The other study investigates the potential of glucose, maltose, fructose and sucrose as external carbon source (Mühling, Belay & Whitton, 2005). The results show that though many *Spirulina* species are able to assimilate glucose and fructose they need a light medium for the assimilation of disaccharides. There are limited data for quality biofuel production under different culture conditions with *Spirulina* sp.

H. pluvialis was cultivated in heterotrophic and mixotrophic cultures with sodium acetate (Orosa, Franqueira, Cid & Abalde, 2005). Reserch demonstrates that 2.5 g/L sodium acetate was detected as optimum concentration; growth was inhibited at higher concentrations. In another study 3 g/L glucose and 2.4 g/L sodium acetate were used as carbon source in the aim of increasing astaxanthin production of *H. pluvialis* (Sun et al., 2015). There are a limited number of studies, especially for the production of astaxanthin, with the use of different carbon sources.

1.3.4 Nutritional and Environmental Conditions for Lipid Production from Microalgae

1.3.4.1 Carbon Source

In cases where external carbon source is used in microbial production environments, carbon source cost corresponds to approximately 50% of the medium cost in algal production (Cheng, Lu, Gao & Wu, 2009). The use of various wastes as a carbon source in the growth medium of microalgae allows economical production and creates an alternative way to waste disposal. The production of valuable by-products that can be used in various industries such as oils, natural dyes, pigments, sugars, antioxidants, bioactive substances in the production process also supports the use of microalgae in biofuel production. Additionally, since the last waste/ pulp

remaining after biofuel production is rich in nitrogen and sulfur, it is suitable for use as fertilizer.

In the selection of organic carbon source to be used in heterotrophic and mixotrophic culture media, cost and absorption and assimilation mechanism of microalgae should be considered. In this respect, glucose is often preferred because of its intracellular transport and assimilation mechanism and higher energy content per mole compared to other substrates (Hamza et al., 2013). Microalgae cells need a lag phase to generate special transport sutructure prior to uptake of carbon sources other than glucose.

Although it is thought that the use of external carbon source may increase the cost of production in heterotrophic and mixotrophic cultures, low or no light intensity need support the economic aspect of the heterotrophic and mixotrophic cultures (Lin & Wu, 2015). Glycerol, which is 10% of transesterification reaction products and therefore waste position, is an important low-cost carbon source alternative (Leite, Paranjape, Abdelaziz & Hallenbeck, 2015). It does not show toxic effects at high concentrations and it is compatible with enzyme and membrane structure, making it suitable for use in microalgal cultures. Crude and technical glycerol contain impurities such as soap, salt and methanol in different proportions. Because of these impurities, it is important to determine the amount of crude or technical glycerol to be used in the microbial growth medium. Although glycerol is used as a raw material in the food industry, a large part of the produced glycerol is considered as waste because it is produced far above the required amount and because the purification procedure of crude glycerol is expensive (Astals, Nolla-Ardèvol & Mata-Alvarez, 2012).

Whey is a side product of cheese production. Approximately 80-90L of whey is formed from cheese produced from 100 L of milk (Božanić, Barukčić & Lisak, 2014; Ghobrini et al. 2020). The composition of the whey may vary depending on the type of cheese. On average, it contains lactose, protein, calcium, phosphate etc. While approximately 70% of the whey is used as raw material in different industries, the remaining part is generally considered as waste. This waste causes significant damage to the environment due to its very high biological oxygen demand (>35,000 ppm) and

chemical oxygen demand (>60,000 ppm) values and it is difficult to clean (Bentahar, Doyen, Beaulieu & Deschênes, 2019; Smithers, 2008). For this reason, it will be beneficial in two ways to transform this industrial waste into a value-added product, especially in the microbial growth medium.

In a study where *Spirulina* sp. was grown in mixotrophic medium containing different concentrations of whey, whey concentration negatively affected the protein content (Pereira et al., 2019). In the same study, the carbohydrate and fat content of the cells increased as the whey concentration increased.

In a study of astaxanthin production by *Heamatococcus pluvialis*, microalgae were grown in photoautotrophic, mixotrophic and heterotrophic cultures (Dechatiwongse & Choorit, 2020). Crude glycerol was used as organic carbon source. Higher biomass yield was obtained in mixotrophic cultivation compared to the other two cultivation types. In addition, it was determined that the amount of biomass increased with increasing glycerol concentration in the mixotrophic culture.

1.3.4.2 Light Intensity

For microalgae, which are photosynthetic organisms, light penetration in the growth medium is an essential growth effect. But when the cells reach the saturation point, light receptors are damaged and photoinhibition may occur (González-Fernández, Sialve, Bernet & Steyer, 2012). The molecular process of photoinhibition displays that the harm caused by the light is caused by the inactivation of the D1 protein of the PSII complex (Singh, Sinha & Hader, 2002). The ability of membrane lipids to desaturate fatty acids is critical for photosynthetic organisms to endure high light stress by stimulate de novo synthesis of D1 protein. The redundant photo-assimilation event in lipid storage from the self-defense process to avert photo-oxidative damage by that, modify excess light energy to chemical energy in the biomolecules.

Light is a source of energy in the photoautotrophic growth phase and microalgae use light energy to convert CO_2 into organic compounds, especially carbohydrates (Juneja, Ceballos & Murthy, 2013). Microalgal growth rate, when saturation reaches

the maximum value of light intensity, the value of light intensity below or above this value is determined to decrease the growth rate (Juneja et al., 2013). A microalga, which is tried to be grown in a mixotrophic medium, tries to adapt to the medium with photoautotrophic metabolism in the first stage and then consumes the carbon source present in the growth medium. This is interpreted as a decrease in the amount of cellular lipid as a result of high light intensity rendering polyunsaturated fatty acids to oxidative stress. It is thought that as light intensity increases, lipid production will increase due to the formation of photo-oxidative stress. This was depicted that electrons formed by reactive oxygen species (ROS) may be depleted in a different pathway rather than lipid synthesis. For example, in plants, oxidative stress is known to cause an increase in soluble sugars. In addition, some studies have shown that low light intensity supports lipid accumulation (Chávez-Fuentes, Ruiz-Marin & Canedo-López, 2018).

In a study examining the effects of glycerol concentration and light intensity on *S. platensis* culture, it was determined that carbohydrate and lipid contents decreased with increasing light intensity (Markou et al., 2019). The protein content, on the other hand, reached the highest value at medium light intensity and decreased at higher light intensity.

H. pluvialis was grown in mixotrophic medium using gluconate as a carbon source and the effect of light intensity was investigated (Pang, Gu, Fu & Chen, 2019). It has been determined that the microalgae adapt to high light intensity and shows high photosynthetic activity. However, it was detected that the number of mobile cells decreased in high light.

1.3.4.3 Nitrogen Concentration

Nitrogen is an essential component of all structural and functional proteins and nucleic acids in cells (Juneja et al., 2013). Inorganic nitrogen taken up into the cell is rapidly assimilated into biochemically active compounds and transferred to the required locations within the cell to meet changing physiological needs. When microalga is in the nutrient-limited medium, it decreases cell division rate and

accumulate carbon source as lipid or starch for energy storage (Shin et al., 2018). The relationship between lipid productivity and nitrogen concentration is species-dependent.

The low nitrogen concentration in the medium triggers lipid production as it increases the C/N ratio (Goncalves, Wilkie, Kirst & Rathinasabapathi, 2016). Similarly, even if the nitrogen concentration in the medium has not changed, lipid production will increase as the increase in carbon amount increases the C/N ratio. Due to there is not enough nitrogen for protein synthesis in the medium, the fixed carbon is used in the synthesis of storage material such as TAG. This mechanism comes to the forefront in mixotrophic production. Carbohydrate will provide both pyruvate as carbon skeleton and ATP and reducing power (NADPH) required for synthesis for lipid synthesis.

In a study examining the production of astaxanthin in different growth media, *H. pluvialis* was grown in media of varying nitrogen concentrations (Wang, Gao, Wu, Huang & Zhang, 2019). It was determined that as the nitrogen concentration increased, the biomass increased, but the astaxanthin content decreased. In a study examining the effect of nitrogen amount on *S. platensis*, biomass productivity was determined to be highest in nitrogen-rich medium (El Baky, El Baroty & Mostafa, 2020). The highest lipid content was obtained in nitrogen limited medium.

1.3.4.4 Phosphorus Concentration

Phosphorus, which is involved in the structure of various energy carrier molecules, phospholipids and nucleic acids, is an important nutrient in microalgae production such as nitrogen and carbon. It takes part in many important bioprocesses such as energy transfer, signal transduction, photosynthesis and respiration (Liang, Zhang, Gu & Cong, 2013).

When the concentration of phosphorus in the growth medium decreases, cell division stops; however, cell growth continues (Wu, Yu & Hu, 2015). When the lipid levels produced depending on changing phosphorus concentration were examined, it

was found that lipid production decreased in parallel with the decrease in phosphorus concentration.

Generally high phosphorus concentration triggers lipid production. Excess electrons deposited from the photosynthetic electron transport chain under environmental stress can trigger the overproduction of ROS. Production of C18 fatty acids results in the use of approximately 24 NADPHs from the electron transport chain, thereby relieving the electron transport chain under stress (Wu et al., 2015). Therefore, the cell may be protected by lipid production from the stress condition created by high phosphorus concentration (Velioglu Tosuner & Ozturk Urek, 2021).

Phosphorus deficiency disrupts the normal function of the phytoplankton cell, so new metabolic pathways are formed. Lipid synthesis is catalyzed by ACCase, which converts acetyl-CoA to malonyl-CoA, and upon completion of the cycle, fatty acids are formed. In the meantime, lipid biosynthesis has been attributed to fatty acids and sn-glycerol-3-phosphate. ACCase generally catalyzes the first reaction of the fatty acid biosynthesis pathway. If there is a lack of phosphorus in the medium, cell division slows down. Excess carbon is absorbed by the cell and enters the Krebs cycle to trigger TAG biosynthesis (Liang et al., 2013).

In a study, the effects of phosphorus concentration on *S. platensis* growth and metabolism were investigated (Chen et al., 2019). While the increase in phosphorus concentration caused an increase in the amount of biomass and protein, it decreased the amount of carbohydrates and did not cause a significant change in the amount of lipids.

In another study examining the effect of phosphorus concentration, it was determined that biomass production decreased when *H. pluvialis* was grown at low or high phosphorus concentrations in basal medium phosphorus concentration (Liyanaarachchi et al., 2020). However, the highest astaxanthin production was obtained in the presence of low phosphorus and light stress condition.

1.3.5 Spirulina sp.

Spirulina sp. are filamentous cyanobacteria that live in aquatic environments with high pH values containing high levels of carbonate and bicarbonate (Vonshak, 1997). Multiple cylindrical cell structures in the open left hand helix structure are the main morphological feature of the genus (Figure 1.6). *S. platensis*, one of the cyanobacteria used in the present thesis study, has found the potential for use in the cosmetic, food and feed industries in terms of its high protein and carotenoid content (Ozturk Urek & Kerimoglu, 2019). It is one of the richest natural antioxidant sources in nature with its minerals such as zinc, manganese, selenium and copper, β-carotene, methionine, vitamin E, vitamins B1 and B6.

Biomass productivity varies between 0.06 and 4.3 g/L.d depending on the species (Mata, Martins & Caetano, 2010). Although 45-70% of dry biomass weight of *Spirulina* sp. is protein, 8-16% carbohydrate and 4-16% lipid, fatty acid composition of total lipids is suitable for biofuel production (Sharma, 2016; Siegenthaler & Murata, 2006). The cell residue formed after the lipids are extracted for biofuel production can be used as raw materials for different industries such as human and animal nutrition and agriculture. In addition, the harvesting of the cells from the growth medium due to their long spiral (20- 100 μm) shapes is economical in terms of energy and cost, and high efficiency can be obtained in separation with easy-to-apply methods.

Figure 1.6 Microscopic vision of *S. plantensis* cell (UTEX collection)

1.3.6 Haematococcus sp.

Another microalga used in the thesis is *Haematococcus pluvialis*, which is a fresh water green microalga that draws attention with its high synthesis of astaxanthin, which is a ketocarotenoid with a wide range of uses in cosmetics, pharmaceuticals, medicine and food industries (Fábregas, Otero, Maseda & Domínguez, 2001). In the life cycle, exhibits four different cell morphologies: zoospor, microzooid, palmella and hematocyst (Figure 1.7). Zoospor, microzooid and palmella phase is called as "green vegetative phase", while hematocyte phase is called as "red stationary phase".

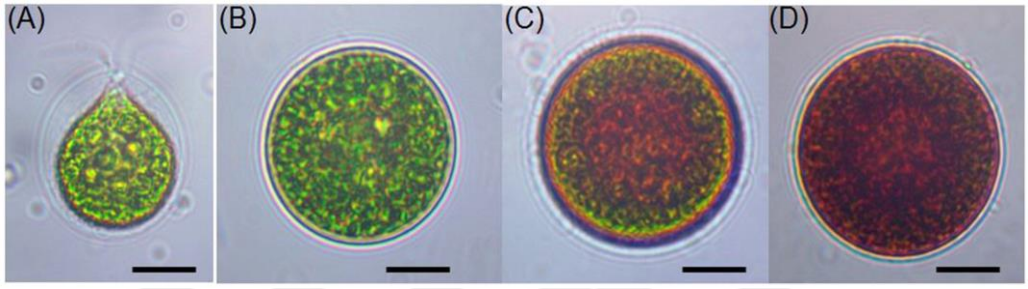

Figure 1.7 Light microscopy images of *H. pluvialis* cells in the life cycle. (A) green vegetative moving cell; (B) green vegetative palmella cell; (C) palmella cell in hematocyte transition; (D) hematocyte cell with astaxanthin accumulation. Scale bar: 10 μm. (Fábregas et al., 2001)

In the vegetative palmella cell, the cell nucleus is located in the center and the cell is enclosed by a thin cell wall. If the cell enters the stress condition, the thickness of the cell wall increases and astaxanthin granules accumulate in the cytoplasm. At the end of this stage, red-brown colored immobile cells are formed, but with the disappearance of the stress factor, these cells appear to return to the mobile, green, vegetative phase (Wayama et al., 2013).

Approximately 30-45% of dry biomass weight is protein, 15-17% is carbohydrate and 20-45% is lipid, but studies on the biofuel potential of *H. pluvialis* have been limited. The properties of fatty acids synthesized by *H. pluvialis* such as high neutral fatty acid ratio and hydrocarbon chain lengths less than C18 is an indication that this microalga is an important potential in biofuel production (Damiani, Popovich, Constenla & Leonardi, 2010). In addition, large cell sizes (5-25 μm) facilitate the departure of the cells from the growth medium, reducing the cost required for

harvesting. Besides lipid production, production of high value-added products synthesized by *H. pluvialis* as a by-product also contributes economically.

1.4 The Aim of This Study

In the thesis study, it is aimed to optimize and increase lipid production efficiency in different environmental/ nutritional conditions by using various industrial wastes (crude/technical glycerol and whey) in varying trophic culture types (photoautotrophic, heterotrophic, mixotrophic) of blue-green (*Spirulina platensis*) and green (*Haematococcus pluvialis*) microalga species. It is also aspired to make the fatty acid content of the produced lipid suitable for high-quality biodiesel production. It is planned to contribute to the creation of new alternatives to economic and renewable energy sources and to increase their use with this thesis.

One of the microalgae (*S. platensis*) used in the thesis study stands out with its protein content, while the other (*H. pluvialis*) draws attention with its high carotenoid content. However, both microalgae have not been adequately evaluated for biofuel production. Glycerol is the final product of the transesterification reaction; it is used being organic carbon source in the microalgal lipid production. The properties of biodiesel such as no toxic effects at high concentrations and compatibility with the enzyme and membrane structure made it suitable for use in microalgal cultures (Damiani et al., 2010).

Another waste used for microalgal lipid production is whey which is also called dairy industry waste. The high amount of organic carbon in these wastes, generated in high volumes, constitutes an important problem in their disposal. For this reason, the evaluation of this high amount of waste as a carbon source in the microalgal growth medium not only provides the potential of the waste as a carbon source but also offers an alternative solution to waste removal.

CHAPTER TWO
MATERIAL AND METHODS

2.1 Culture Conditions of Microalgae

The microalgae *H. pluvialis* CCAP 34/12 and *S. platensis* (Gomont) 1892 were supplied from Cukurova University, Faculty of Aquaculture, Turkey. For the sustenance of *H. pluvialis* microalgae has been growth in Bold Basal Medium (BBM) under photoautotrophic culture for the sustenance (Table 2.1) (Janet, Stein & Stein, 1973). In addition, the most suitable basal environment was determined by growing the *H. pluvialis* in BG-11 medium at the first stage (Stanier, Kunisawa, Mandel & Cohen-Bazire, 1971). The cultivation was carried out in 1 L serum bottle with 750 mL growth medium at 2500 lux (33.75 µmol photon m^{-2} s^{-2}) white light power, pH 7.0 and 28°C and the cultures were mixed by continuous filtered aeration.

S. platensis cyanobacteria has been growth in Zarrouk's medium (Table 2.2) For the sustenance under photoautotrophic culture (Zarrouk, 1966). The cultivation was performed in 1 L serum bottle with 750 mL growth medium at 2500 lux (33.75 µmol photon m^{-2} s^{-2}) white light power, pH 9.0 and 30°C and the cultures were mixed by continuous filtered aeration.

Figure 2.1 *H. pluvialis* (left) and *S. platensis* culture (right)

Table 2.1 The contents of BBM

Component	**Amount (g/L)**	**Components of Trace Element Solution**	**Amount (g/50 mL)**
$NaNO_3$	0.25	$MnCl_2.4H_2O$	0.072
KH_2PO_4	0.175	$ZnSO_4.\ 7H_2O$	0.441
K_2HPO_4	0.75	$Na_2(MoO_4)_2.2H_2O$	0.0355
$MgSO_4.7H_2O$	0.75	$CuSO_4.5H_2O$	0.0785
$CaCl_2.2H_2O$	2.5	$Co(NO_3)_2.6H_2O$	0.0245
NaCl	2.5	**Components of EDTA Solution**	**Amount (g/50 mL)**
H_3BO_3		Na_2EDTA	2.5
Trace element solution	1 mL	KOH	1.55
EDTA solution	1 mL	**Components of Acidified Iron Solution**	**Amount (g/50 mL)**
Acidified iron solution	1 mL	$FeSO_4.7H_2O$	0.249
		H_2SO_4	20 µL

Table 2.2 The contents of Zarrouk's Medium

Component	**Amount (g/L)**	**Components of Trace Element Solution**	**Amount (g/L)**
$NaHCO_3$	18	H_3BO_3	2.86
$NaNO_3$	2.5	$MnCl_2.4H_2O$	1.8
K_2SO_4	1	$ZnSO_4.7H_2O$	0.22
NaCl	1	$CuSO_4$	0.08
K_2HPO_4	0.5	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.02
$MgSO_4.7H_2O$	0.2		
Na_2EDTA	0.08		
$CaCl_2$	0.04		
$FeSO_4.7H_2O$	0.01		
Trace element solution	1 mL		

2.2 Optimization of Microalgae Growth Conditions

In the mixotrophic and heterotrophic cultures glycerol (crude and technical) and whey were used as external carbon sources. Waste (crude) and technical (partially refined) were obtained from DP Tarım Enerji (Torbalı-İzmir) and they were 54.35% and 82.3% purity, respectively. Content of crude and technical glycerol are given in Table 2.3. Whey was supplied by Balkan Dairy Products (Torbalı-İzmir).

Mixotrophic and heterotrophic cultivations were achieved in BBM or Zarrouk's medium that included various concentration of crude or technical glycerol (1, 10 and 30 mM) or whey (1, 10 and 30%, v/v) being carbon source. The beginning optical densities of cultures were adjusted to 0.2 (OD= 680 nm for *H. pluvialis*, OD= 600 nm for *S. platensis*). Batch cultivation was carried out in 250 mL Erlenmeyer with 100 mL growth medium at 100 rpm. The white fluorescent lamps (1500 lux= 20.25 µmol photon m^{-2} s^{-2}) were used for continiuous brightness in mixotrophic cultures while heterotrophic cultures were carried out in totally dark. The external conditions are adjusted as pH 7.0 and 28°C *H. pluvialis* and pH 9.0 and 30°C for *S. platensis*.

After determining the optimum carbon source type and concentration for lipid production, the optimum nitrogen concentration (0, 1.5 and 2.9 mM), light intensity (0, 1500, 2500 and 4000 lux) and phosphorus concentration (0, 2.5 and 5.6 mM) for *H. pluvialis* were determined. Similarly, after determining the appropriate carbon source and concentration for optimum lipid production of *S. platensis*, the optimum values of nitrogen (0, 15 and 29 mM), phosphorus (0, 1.5 and 2.9 mM) concentrations and light intensity (0, 1500, 2500 and 4000 lux) were determined.

Specific growth rate (µ) was adjusted in line with Equation 2.1 according to OD levels (Kong et al., 2013). (X: OD level t: time)

$$\mu = \ln \frac{X1 - X0}{t1 - t0} \tag{2.1}$$

Table 2.3 The content of crude and technical glycerol (DB Tarımsal Enerji, 2021)

	Crude glycerol	**Technical glycerol**
Glycerol (%)	54.35	82.3
Water (%)	34.81	7.7
Soap (%)	6.52	6.6
NaOH (%)	3.53	2.4
NaCl (%)	0.64	0.5
Methanol (ppm)	55	102

2.3 Investigation of Intracellular Compound Levels According to Incubation Period

2.3.1 Investigation of Protein Content

The microalgae cells were homogenized with 50 mM, pH 7.0 phosphate buffer and cell debris were removed by centrifugation (12000 rpm, 10 min, 4°C), the supernatant was used for determination of protein level (Esen & Ozturk Urek, 2015). The homogenization process was optimized for *S. platensis* at 1 minute 8000 rpm and 1 minute 9500 rpm, while for *H. pluvialis* 1 minute 8000 rpm, 1 minute 9500 rpm and 1 minute 13500 rpm.

Protein content assay was achived by the Bradford (1976) method. Bovine serum albumin (0-250 ppm) was used as standard. Coomassie Brillant Blue G-250 (100 mg) was dissolved in ethanol (50 mL) for the reagent and 85% phosphoric acid (100 mL) was added, lastly the total volume of 1000 mL was completed with water. For the assay 100 µL of analyte was mixed with 900 µL of reagent. Arter incubated at room temperature for 2 minutes the absorbance was detected at 595 nm. The standard function was $y = 0.00637x$ ($R^2 = 0.986$).

2.3.2 Determination of Chlorophyll Content

Chlorophyll levels were detected as explaned by Lichtenthaler & Wellburn (1983). 5 mL of microalgal cell was collected by centrifugation at (5000 rpm, 10 minutes).

The *H. pluvialis* pellet was homogenized with ethanol (5 mL) at 8000, 9500 and 13500 rpm for total 3 minutes with 30 seconds breaks. The *S. platensis* pellet was homogenized with ethanol (5 mL) at 8000 and 9500 rpm for total 2 minutes with 30 seconds breaks. After removing of cell residue by centrifugation the obtained supernatant was measured at 664.2 and 648.6 nm. Chlorophyll a and b levels were adjusted in line with equations 2.2 and 2.3.

$$\text{Chl a} = 13.36 \times \text{Abs}_{664.2} - 5.19 \times \text{Abs}_{648.6} \quad (2.2)$$

$$\text{Chl b} = 27.43 \times \text{Abs}_{648.6} - 8.12 \times \text{Abs}_{664.2} \quad (2.3)$$

2.3.3 Determination of Total Carbohydrate Content

Total carbohydrate level was determined by phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). After homogenizing as described in section 2.3.2, the resulting homogenate was used in the total carbohydrate analysis. 1 mL growth medium supernatant and 5% (w/v) phenol solution (1 mL) are mixed and concentrated sulphuric acid (5 mL) is added to the mixture. The mixture was incubated at room temperature for 20 minutes and the absorbance (470 nm) is detected. Glucose solution (0- 250 μg/mL) is used as standard, $y = 0.00762x$ ($R^2 = 0.998$).

2.3.4 Determination of Total Lipid Content

Total lipid level was determined according to method of Mishra et al., (2014). Vanillin (0.6 g) was dissolved in ethanol (10 mL) and distilled water (90 mL) and phosphoric acid (400 mL) were added to the mixture. Microalgea sample (100 μL) was mixed with concentrated H_2SO_4 (2 mL) and incubated at 100°C for 10 minutes. After cooling the mixture in ice bath for 5 minutes, phospho-vanillin reagent (5 mL) was added and the mixture incubated at 37°C for 15 minutes. The absorbance (530 nm) was detected. Sunflower seed oil (0- 100 μg/mL) was used as standard and the function was $y = 0.00453x$, ($R^2 = 0.993$). Lipid producvitiy was detected in line with equation 2.4.

$$\text{lipid productivity} = \frac{\text{total lipid (mg)}}{\text{growth medium volume (L) x incubation time (day)}} \quad (2.4)$$

2.3.5 Determination of Proline Content

The proline content of produced cells in control and optimized medium were detected according to Bates, Waldren & Teare (1973). After centrifugation 1 g cell and 10 mL of 3% sulfosalicylic acid were mixed and cells were homogenized. The supernatant (2 mL) were mixed with 2 mL of ninhydrine and 2 mL of acetic acid. The mixture was incubated in boiling water bath for 1 hour and then for cooling the mixtture tubes were immerged to the ice bath. After adding 4 mL of toluen the absorbance was measured at 520 nm. Proline was used as standard and the function was $y = 0.0241x$, ($R^2 = 0.986$).

2.3.6 Determination of Lipid Peroxidation (LPO)

The LPO values of produced cells in control and optimized medium were detected according to Ohkawa, Ohishi & Yagi (1979). The harvested cells (0.1 g) by centrifugation were mixed with 1.5 mL of 5% trichloroacetic acid (TCA) and the cells were homogenized. The supernatant and 0.5% thiobarbituric acid (TBA) were mixed in equal volumes. The TBA solution was prepared in 20% of TCA. The mixture was incubated in water bath (95°C) for 30 minutes and then for cooling the mixture tubes were immerged to the ice bath. The absorbance (532 and 600 nm) is detected. The concentration of malondialdehyde (MDA) in the cell extract was adjusted according to equation 2.5.

$$\text{MDA} = \frac{(A532 - A600)\ x\ volume\ of\ extraction}{155\ x\ g\ sample} \quad (2.5)$$

2.4 Investigation of Variation in Extracellular Liquid Components Depending on Incubation Period

The supernatant obtained after separation of cells from the growth medium by centrifugation (12000 rpm, 10 min, 4°C) was used in the following assays.

2.4.1 Determination of Total Carbohydrate Content

Total carbohydrate content was determined according to phenol-sulfuric acid as described in section 2.3.3.

2.5 Lipid Extraction and Characterization Produced Under Optimum Conditions

Two different methods have been tried for extraction of lipid and FAME production. In the first method, the collected cells, purified water (0.8 mL), methanol (2 mL) and chloroform (1 mL) were mixed as far as single phase was obtained. Prufied water (2 mL) and chloroform (2 mL) were also added to obtain a biphasic mixture (Mohammad Mirzaie, Kalbasi, Mousavi & Ghobadian, 2016). The extracted and dried lipid (100 mg) was dissolved in hexane (10 mL) and was mixed with 2 N KOH (100 μL). The supernatant was used for FAME anaylsis by gas chromatography (Agilent 7890 GC, 30 m capillary column, FID, He gas).

In the second method, the cells collected by centrifugation were lyophilized. Direct FAME production was performed using dry cell (Indarti, Majid, Hashim & Chong, 2005). 15 mg lyophilized cell and 3 mL solution (methanol: H_2SO_4: chloroform, 1.7:0.3:2, v/v) were mixed and incubated at 100°C for 30 min. After cooling to room temperature, 1 mL of purified water was added to the mixture and vortexed. The heavier phase from the two phases formed was transferred to a tube and dried in the oven. The fatty acid composition of the produced lipid was detected by GC after adding 1 mL hexane to dried lipid.

For FAME analysis, flame ionization detector and capillary column (60 m x 0.25 mm) were used. The carrier gas, He, flowed at 1.3 mL/min flow rate. The injector temperature was 250°C and the detector temperature was 270°C. The oven temperature was fixed to 175°C for 10 minutes, and then reached 220°C with 3°C/min raising rate and stand at that temperature for 5 minutes. Commercial fatty acid mixture (FAME MIX/ C14-C22- Sigma) was used as standard.

2.6 Biodiesel Potential of Produced FAME

The formulas (2.6- 2.11) were used to figure out the biofuel capability of the produced lipid (Rós et al., 2013).

- Iodine value:

$$\mathrm{IV} = \frac{\sum(254 \times \mathrm{D} \times \mathrm{N})}{\mathrm{M}} \quad (2.6)$$

(D: Number of double bonds; M: molecular weight; N: percentage of each fatty acid component).

- Cetane number: It is accepted as an indicator of the ignition quality of fuels.

$$\mathrm{CN} = 46.3 + \frac{5458}{\mathrm{SV}} - (0.224 \times \mathrm{IV}) \quad (2.7)$$

- Saponification value:

$$\mathrm{SV} = \frac{\sum(560 \times \mathrm{N})}{\mathrm{M}} \quad (2.8)$$

- Degree of unsaturation and long chain saturated factor:

$$\mathrm{DU} = (\%\ \text{Monounsaturated fat weight}) + 2 \times (\%\ \text{polyunsaturated fat weight}) \quad (2.9)$$

$$\mathrm{LCSF} = (0.1 \times \mathrm{C16}) + (0.5 \times \mathrm{C18}) + (1 \times \mathrm{C20}) + (1.5 \times \mathrm{C22}) + (2 \times \mathrm{C24}) \quad (2.10)$$

- Oxidative stability:

$$\mathrm{OS} = -(0.0384 \times \mathrm{DU}) + 7.77 \quad (2.11)$$

2.7 Statistical Analysis

All experiments were carried out in triplicates (n=3) and repeated 3 times. Each value is an average of 3 parallel replicates. Data were presented as mean ± standard deviation andit was analyzed by analysis of variance (ANOVA) to identify the

significantly different groups at ($p<0.05$) by one-way ANOVA test using SPSS, statistical analysis software package (SPSS for windows ver. 21.00, USA). Also, the data were examined by Tukey' HSD test to identify the difference among multiple variables (SPSS for windows ver. 21.00, USA). Pearson's correlation was used for corelation analysis.

CHAPTER THREE
RESULTS AND DISCUSSION

In the first stage of the study, both microalgae species were grown in a photoautotrophic media and their chlorophyll, protein, total carbohydrate and total lipid contents were determined depending on incubation period. Photoautotrophic cultivation was used as a control condition. Photoautotrophic culture is commenly used cultivation type because of its easy application conditions. In the next step, different concentrations of organic carbon sources (crude glycerol, technical glycerol and whey) were first tested in mixotrophic and heterotrophic cultures of *H. pluvialis* and *S. platensis*. In mixotrophic conditions swift growth rates and great lipid productivity are detected. Similarly some microalgea type shows higher lipid prodctivity in heterotrophic conditions. Glycerol and whey are appropriate carbon and energy sources in mixotrophic and heterotrophic cultures due to their high amount and rich content. After determining the appropriate trophic condition and carbon source type and concentration for lipid production, the effects of nitrogen, phosphorus concentration and light intensity were investigated. The lipid produced under optimum conditions was extracted using two different methods and the fatty acid profile was determined by GC. Biofuel properties such as CN, IV, OS of the lipid produced were determined by using the fatty acid profile and compared with European and American standards.

3.1 Photoautotrophic Cultivation of *H. pluvialis* and *S. platensis*

Two different compositions have been tried in *H. pluvialis* growth medium which were BBM and BG-11. When the specific growth rates were calculated considering the growth curves (Figure 3.1), higher specific growth rate was observed in the BBM (0.135 day^{-1}). For this reason, *H. pluvialis* production continued with BBM. Growth curves, chlorophyll, protein, carbohydrate and lipid contents were determined depending on the incubation period by growing *S. platensis* in Zarrouk medium and *H. pluvialis* in BBM. These cultivations were considered as control conditions in the study.

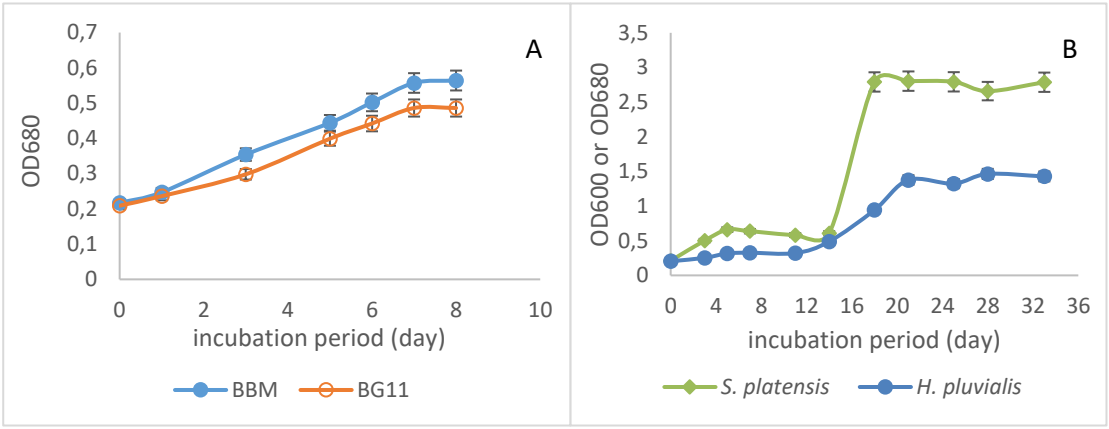

Figure 3.1 (A) The growth curves of *H. pluvialis* in BBM and BG-11 media, (B) The growth curves of *H. pluvialis* and *S. platensis* in photoautotrophic cultures

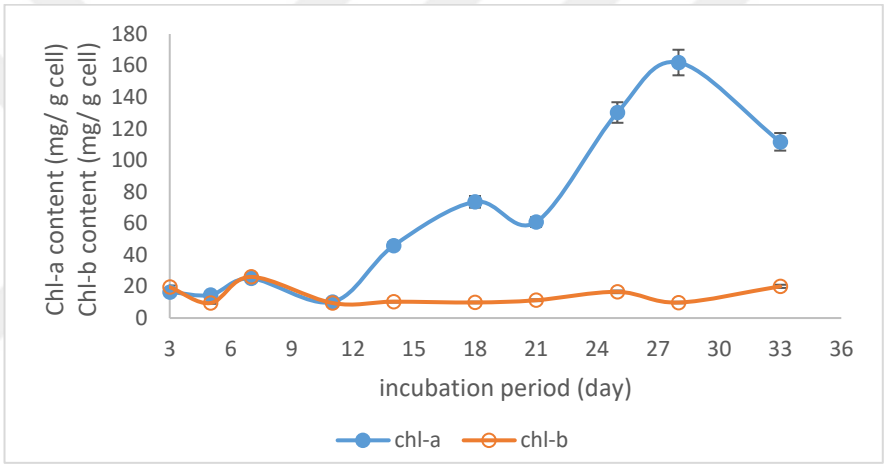

Figure 3.2 Chlorophyll a and b content changes of *H. pluvialis* in photoautotrophic culture

The specific growth rate of *H. pluvialis* grown in photoautotrophic condition was calculated as 0.15 day^{-1}. In this medium, it was found that the amount of chlorophyll a and b increased during the incubation (Figure 3.2). The maximum amount of chlorophyll a (161.93±0.76 mg/ g cell) was detected on the 28th day of incubation. The maximum lipid content was determined as 0.99±0.04 mg/ g cell on the 28th day of incubation (Figure 3.3). The fact that the maximum lipid content was reached on the day when the maximum chlorophyll content was reached, but the total amount of carbohydrate was low, can be interpreted as microalgae stores excess carbon and energy as lipid. While the maximum protein content (0.49±0.03 mg/ g cell) was

detected on the 25th day of incubation, the maximum total carbohydrate content (0.36±0.02 mg/ g cell) was detected on the 11th day of incubation (Figure 3.3).

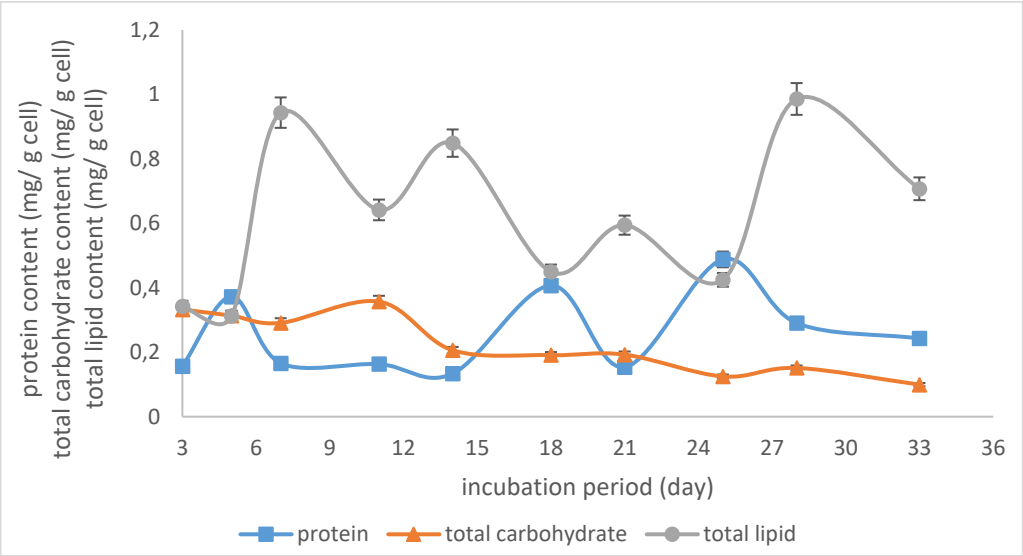

Figure 3.3 Protein, total carbohydrate and total lipid content changes of *H. pluvialis* in photoautotrophic culture

The specific growth rate of *S. platensis* grown in photoautotrophic condition was calculated as 0.39 day^{-1}. Chlorophyll a and b amounts showed an increasing trend in the first days of incubation, and drops were determined in the last days (Figure 3.4). The maximum amount of chlorophyll a was determined as 375.96±1.73 mg/ g cell on the 25th day of incubation. While the amount of lipid decreased during the incubation period, a fluctuation was detected in the amount of protein and carbohydrate (Figure 3.5). The maximum lipid content was determined as 4.85±0.22 mg/g cell on the 3rd day of incubation. While the maximum protein content (1.31±0.05 mg/ g cell) was determined on the 33th day of incubation, the maximum total carbohydrate content (0.67±0.03 mg/ g cell) was detected on the 14th day of incubation (Figure 3.5).

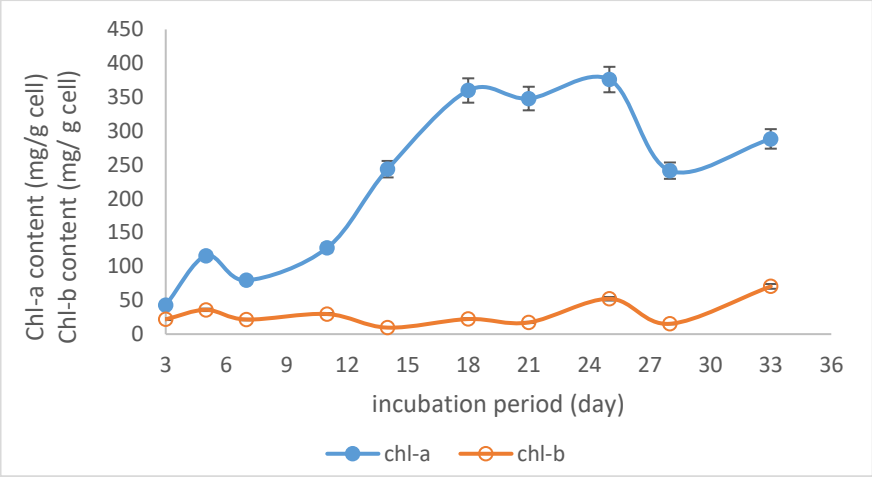

Figure 3.4 Chlorophyll a and b content changes of *S. platensis* in photoautotrophic culture

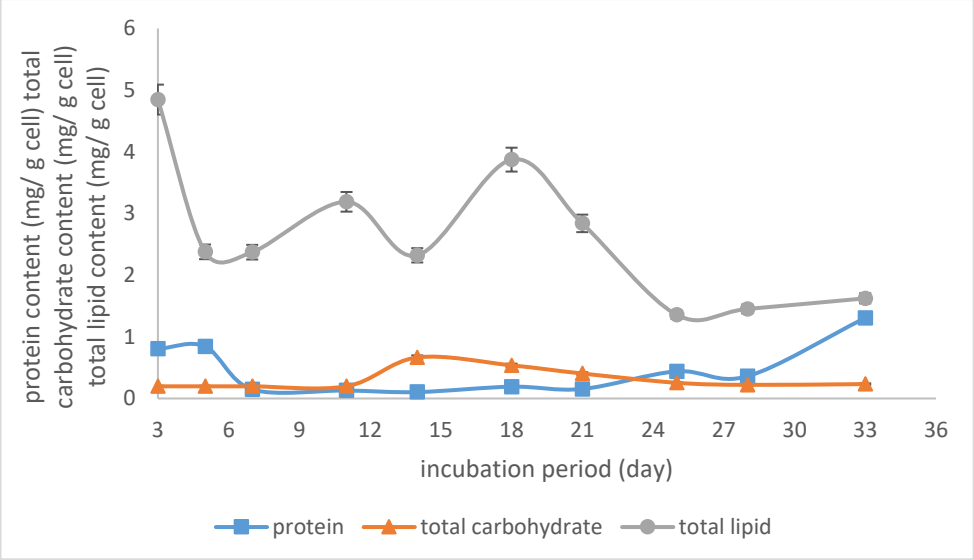

Figure 3.5 Protein, total carbohydrate and total lipid content changes of *S. platensis* in photoautotrophic culture

3.2 Determination of Appropriate Tropic Condition and Carbon Source for *H. pluvialis*

3.2.1 Growth of H. pluvialis in the Presence of Crude Glycerol in Mixotrophic and Heterotrophic Cultures

When the growth curves of *H. pluvialis* grown in mixotrophic or heterotrophic cultures containing crude glycerol in different concentrations (1, 2.5 and 10 mM) are examined it is observed that microalgae grow better in mixotrophic cultures, whereas there is no significant difference between them in terms of growth in heterotrophic cultures ($p<0.05$) (Figure 3.6). The maximum OD value (0.54 ± 0.02 on 25^{th} day) was detected in the mixotrophic culture containing 1 mM crude glycerol, and at the same time, the maximum specific growth rate ($\mu= 0.09$ day^{-1}) was determined in this growth medium ($p<0.05$). It has been determined in studies that *H. pluvialis* grows very slowly under heterotrophic conditions, and this situation can be interpreted as that light is essential for the growth of *H. pluvialis* (Minhas, Hodgson, Barrow & Adholeya, 2016; Andrulevičiūtė, Makarevičienė, Skorupskaitė & Gumbytė, 2014).

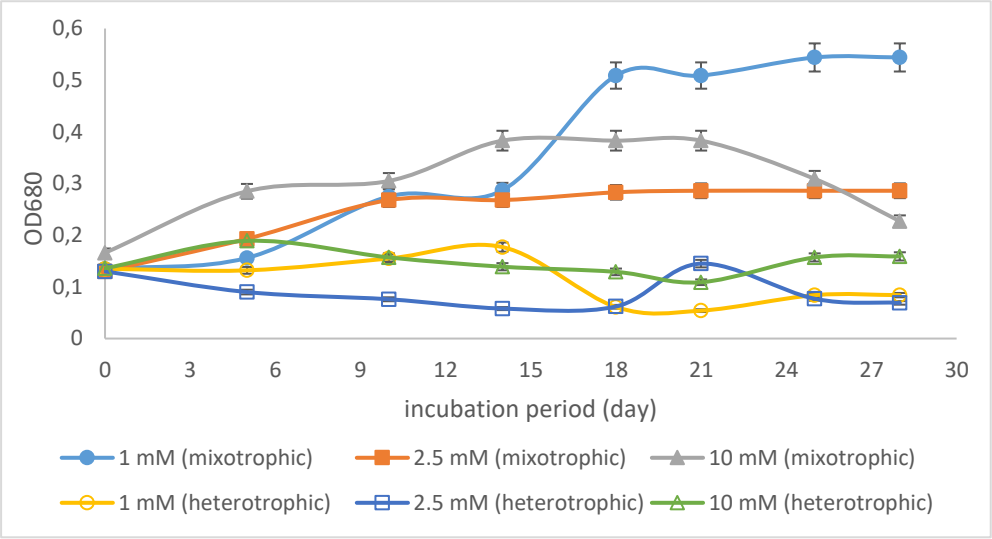

Figure 3.6 OD values of *H. pluvialis* in mixotrophic and heterotrophic cultures in the presence of crude glycerol at varying concentrations (1, 2.5 and 10 mM)

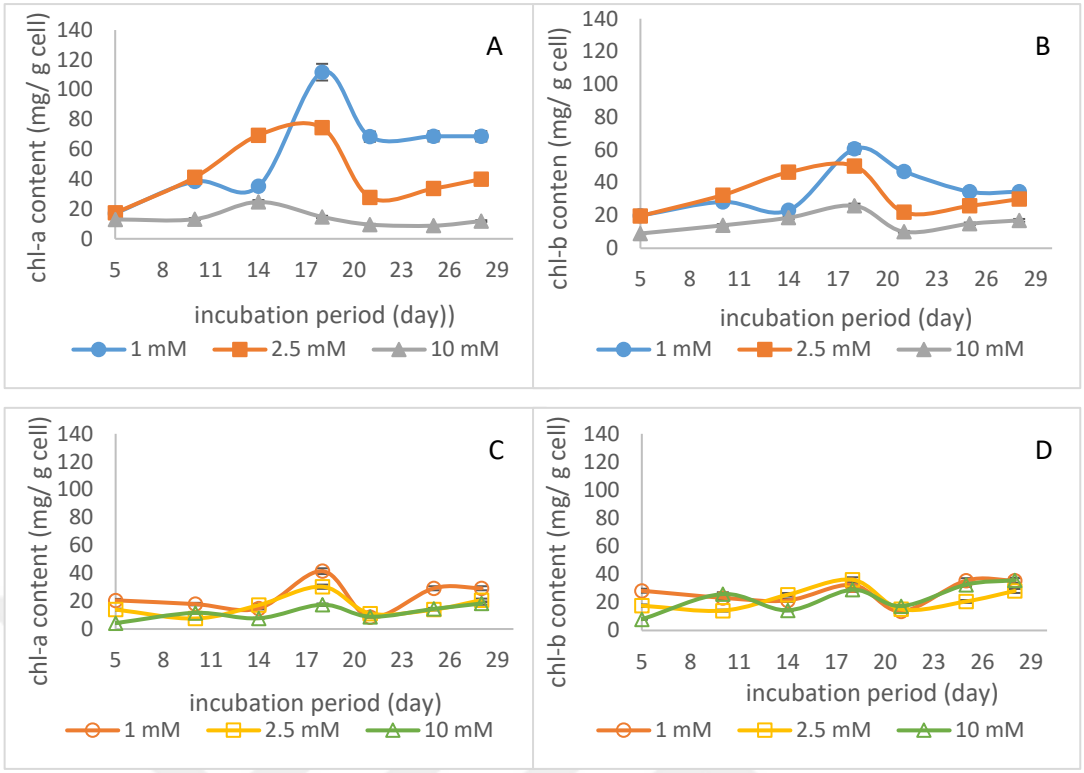

Figure 3.7 Chlorophyll a and chlorophyll b content of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing crude glycerol at varying concentrations (1, 2.5 and 10 mM) (A) Chlorophyll a in mixotrophic culture, (B) Chlorophyll b in mixotrophic culture, (C) Chlorophyll a in heterotrophic culture, (D) Chlorophyll b in heterotrophic culture

When chlorophyll change is examined during the incubation period, chlorophyll a values appear to be higher in mixotrophic cultures than in heterotrophic cultures (Figure 3.7). The maximum chlorophyll a (111.75±0.53 mg/ g cell) and chlorophyll b (60.62±0.28 mg/ g cell) values were detected on the 18th day of incubation in mixotrophic culture including 1 mM crude glycerol ($p<0.05$). In mixotrophic cultures, it is expected that the amount of chlorophyll decreases as the organic carbon source concentration increases. Because microalgae will use the organic carbon source in the growth medium, it will need less photosynthesis.

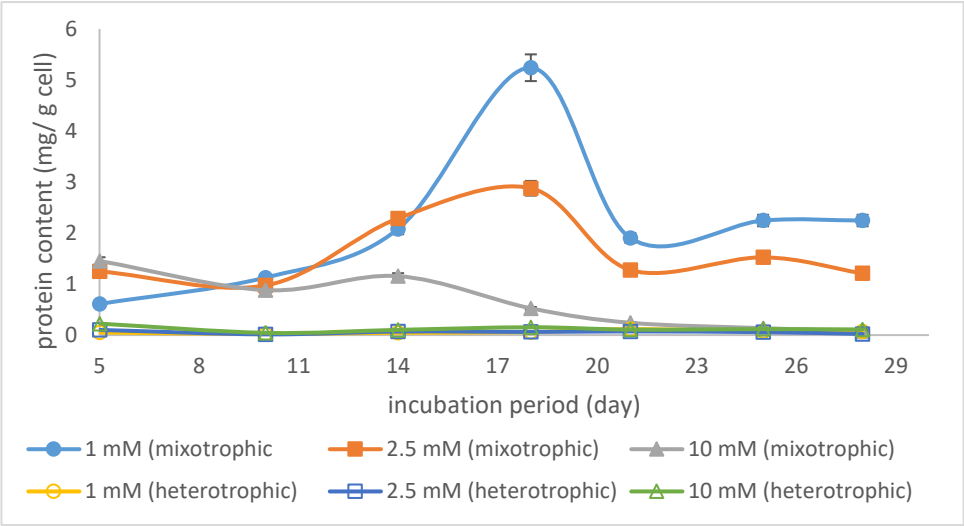

Figure 3.8 Protein content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing crude glycerol at varying concentrations (1, 2.5 and 10 mM)

Protein values in mixotrophic cultures containing crude glycerol are higher than those in heterotrophic cultures (Figure 3.8). This indicates that the heterotrophic culture is not relevant for the growth of *H. pluvialis*. The maximum protein value (5.24±0.21 mg/ g cell) was detected on the 18th day of incubation in mixotrophic culture including 1 mM crude glycerol ($p<0.05$). In the mixotrophic medium, the protein value decreased as the organic carbon source concentration increased. When this result is combined with the amount of chlorophyll, it can be interpreted that the suitable growth medium for *H. pluvialis* is mixotrophic cultures with low carbon source.

While total carbohydrate values were determined higher in mixotrophic cultures, no significant change was detected in heterotrophic cultures (Figure 3.9). The maximum total carbohydrate value (494.6±2.35 mg/ g cell) was detected on the 18th day of incubation in mixotrophic culture including 1 mM crude glycerol ($p<0.05$). Similar to protein and chlorophyll amounts, carbohydrate values were lower in mixotrophic cultures with high glycerol.

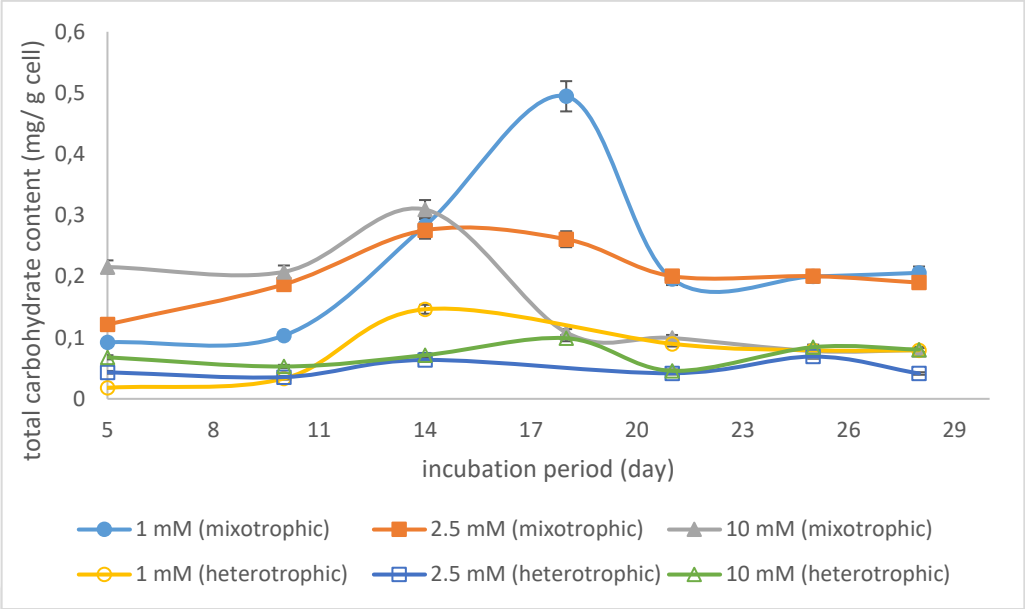

Figure 3.9 Total carbohydrate content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing crude glycerol at varying concentrations (1, 2.5 and 10 mM)

When the total lipid changes during the incubation period of *H. pluvialis* grown in growth media containing crude glycerol was examined while higher lipid levels were achieved in cultures containing 1 and 2.5 mM crude glycerol, it appears to remain lower in other cultures (Figure 3.10). The maximum lipid level (4.32±0.22 mg/ g cell) was detected on the 14th day of incubation in mixotrophic culture including 2.5 mM crude glycerol ($p<0.05$). This value is approximately 4.5 times higher than the lipid value produced under control conditions. It is also an important advantage that this high lipid value is reached earlier in the incubation days compared to the control condition. When the glycerol concentration exceeds 2.5 mM in crude glycerol containing media, the amount of lipid decreases because the components of the glycerol, such as soap, salt and methanol, were increased. In heterotrophic culture, on the other hand, high lipid content could not be obtained because, in general, adaptation to the heterotrophic environment was not observed.

In a similar study, the change in lipid and astaxanthin contents was investigated by growing *H. pluvialis* in mixotrophic cultures containing crude glycerol at different concentrations (0.5-1 mL/L) (Zhang, Zhang, Liu & Yang, 2020). It was determined that glycerol concentration did not cause a change in biomass and chlorophyll content.

However, it was determined that the amount of lipid increased with the increasing amount of glycerol. This was interpreted as increasing the amount of acetyl-CoA by converting the glycerol taken into the cell from the growth medium to glyceraldehyde-3-phosphate. Thus, lipid production was triggered by stimulating the fatty acid pathway.

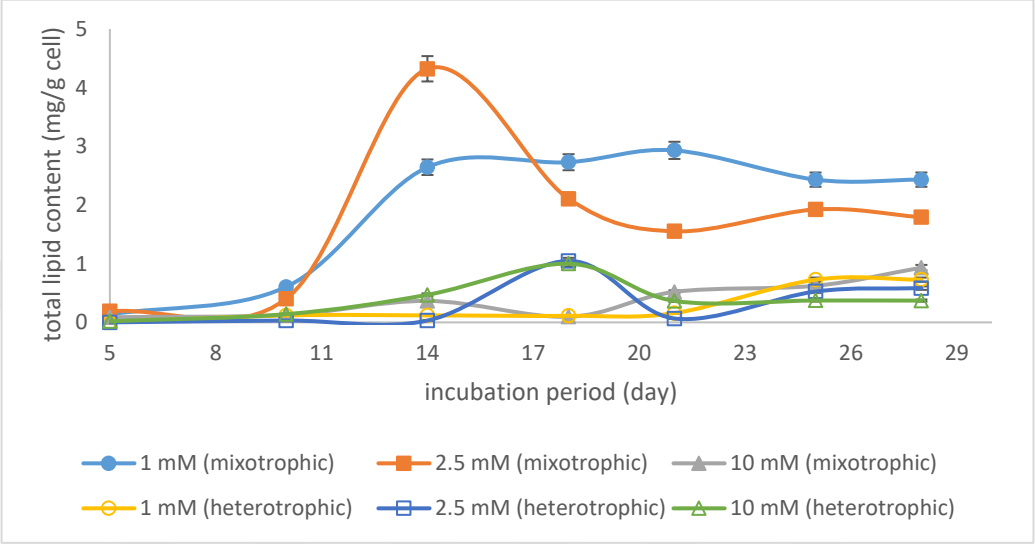

Figure 3.10 Total lipid content changes of *H. pluvialis* cultures in mixotrophic and heterotrophic cultures in the presence of crude glycerol at varying concentrations (1, 2.5 and 10 mM)

3.2.2 Growth of H. pluvialis in the Presence of Technical Glycerol in Mixotrophic and Heterotrophic Cultures

When the growth curves of *H. pluvialis* grown in mixotrophic or heterotrophic cultures containing technical glycerol in different concentrations (1, 2.5 and 10 mM) were examined it was determined that microalgae grew better in mixotrophic cultures, but did not grow in heterotrophic cultures (Figure 3.11). Although the maximum OD value (1.32±0.54) was reached on the 21st day of incubation in mixotrophic medium containing 2.5 mM technical glycerol, the maximum specific growth rate (μ= 0.11 day^{-1}) was detected in 1 mM technical glycerol containing mixotrophic medium ($p<0.05$). In a similar study, it was determined that the medium where *H. pluvialis* showed the highest growth was containing technical glycerol (Dominguez-Bocanegra, Legarreta, Jeronimo & Campocosio, 2004). In this study, it was determined that the specific

growth rate of *H. pluvialis* in the culture where crude glycerol is used is lower than the medium in which technical glycerol is used.

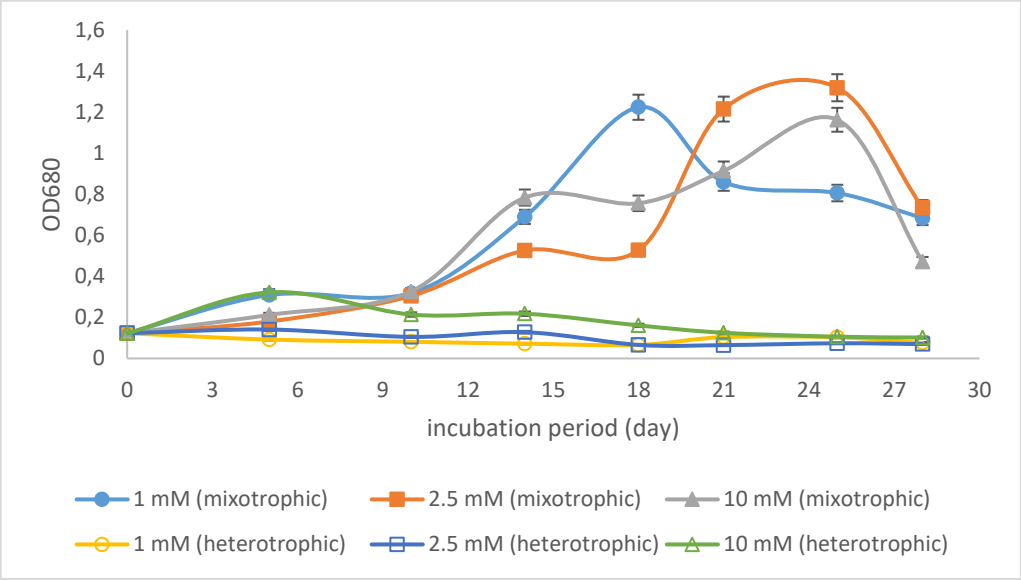

Figure 3.11 OD values of *H. pluvialis* cultures in mixotrophic and heterotrophic cultures in the presence of technical glycerol at varying concentrations (1, 2.5 and 10 mM)

When chlorophyll levels were examined during the incubation period, high chlorophyll a values were reached in mixotrophic cultures, while no significant changes were detected in chlorophyll values during incubation in heterotrophic cultures (Figure 3.12). The maximum chlorophyll a (97.51±0.45 mg/ g cell) and chlorophyll b (50.02±0.23 mg/ g cell) values were detected on the 14th day of incubation in mixotrophic culture including 2.5 mM technical glycerol ($p<0.05$). In the mixotrophic culture including low concentration of glycerol, the amount of chlorophyll was high in the first days of incubation, but decreased in the last days. On the other hand, the situation is the opposite in the medium with high concentration of glycerol. These results can be interpreted as photosynthetic metabolism is more active when the beginning carbon source concentration is low. When the begining carbon source concentration is high, the microalgal cell may first be using the heterotrophic metabolism more actively.

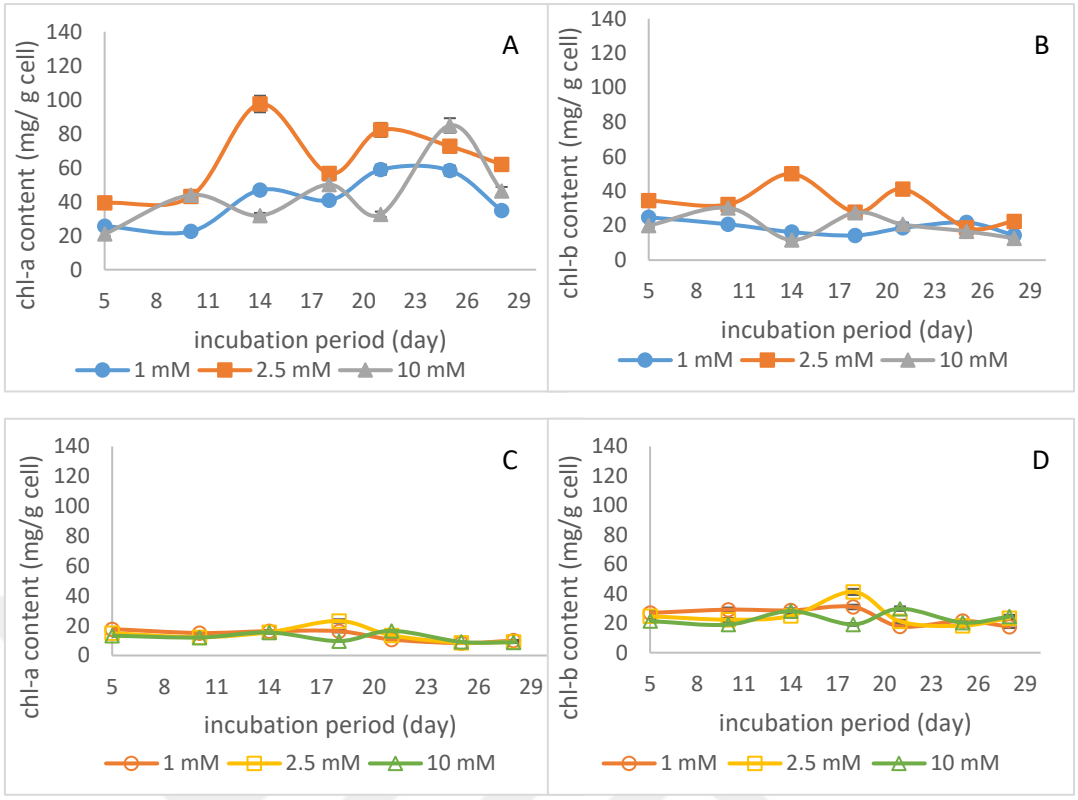

Figure 3.12 Chlorophyll a and chlorophyll b content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing technical glycerol at varying concentrations (1, 2.5 and 10 mM) (A) Chlorophyll a in mixotrophic culture, (B) Chlorophyll b in mixotrophic culture, (C) Chlorophyll a in heterotrophic culture, (D) Chlorophyll b in heterotrophic culture

The maximum protein value (1.49±0.06 mg/ g cell) was detected on the 14th day of incubation in 2.5 mM glycerol mixotrophic medium, while protein values in heterotrophic cultures remained at low level ($p<0.05$) (Figure 3.13). It is expected that protein levels are low in heterotrophic cultures when considering the microbial growth curves of *H. pluvialis*.

When the total carbohydrate change in the growth medium was examined, total carbohydrate values showed a similar trend in mixotrophic and heterotrophic cultures (Figure 3.14). The maximum values were found on 14th day of incubation in mixotrophic medium (0.28±0.01 mg/ g cell) containing 2.5 mM technical glycerol and in heterotrophic medium containing 10 mM technical glycerol (0.29±0.01 mg/ g cell) was detected on the 25th day of incubation ($p<0.05$). In heterotrophic culture, higher

carbohydrate values were achieved at higher carbon concentration, because only heterotrophic metabolism is active in this cultivation.

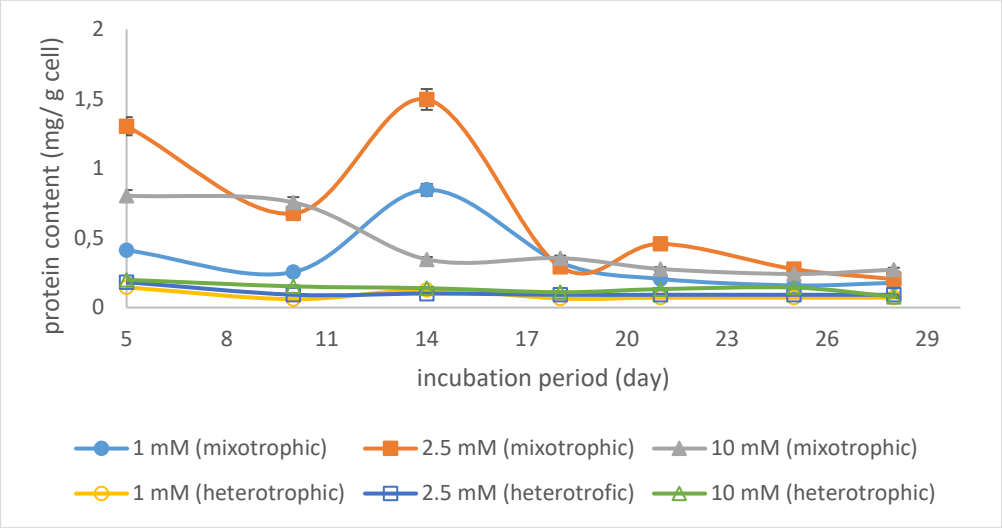

Figure 3.13 Protein content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing technical glycerol at varying concentrations (1, 2.5 and 10 mM)

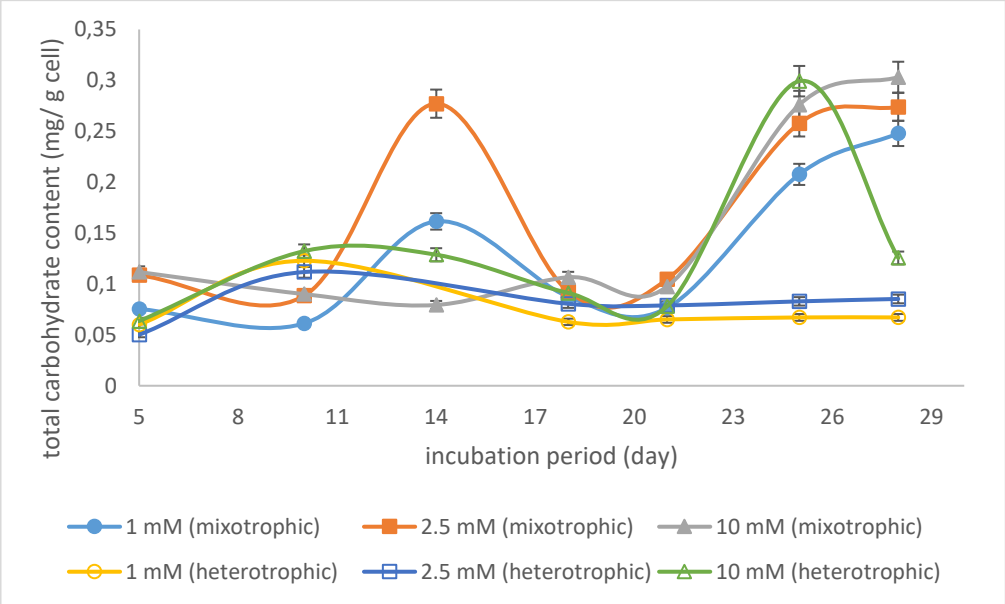

Figure 3.14 Total carbohydrate content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing technical glycerol at varying concentrations (1, 2.5 and 10 mM)

When the total lipid changes of *H. pluvialis* grown in technical glycerol-containing growth media were examined during the incubation period, high values were reached

in mixotrophic cultures (Figure 3.15). The maximum values obtained were 1.22±0.05 mg/ g cell and 1.14±0.02 mg/ g cell, in 10 mM mixotrophic (5th day), 2.5 mM mixotrophic cultures (5th day), respectively ($p<0.05$). In the technical glycerol containing medium, the amount of lipid produced remains low than crude glycerol because some substances such as methanol contained in the technical glycerol may inhibit lipid production. High concentration of methanol caused toxic effect for *Chlorella* sp. in similar way (Choi et al., 2011; Kotzabasis, Hatziathanasiou, Bengoa-Ruigomez, Kentouri & Divanach, 1999).

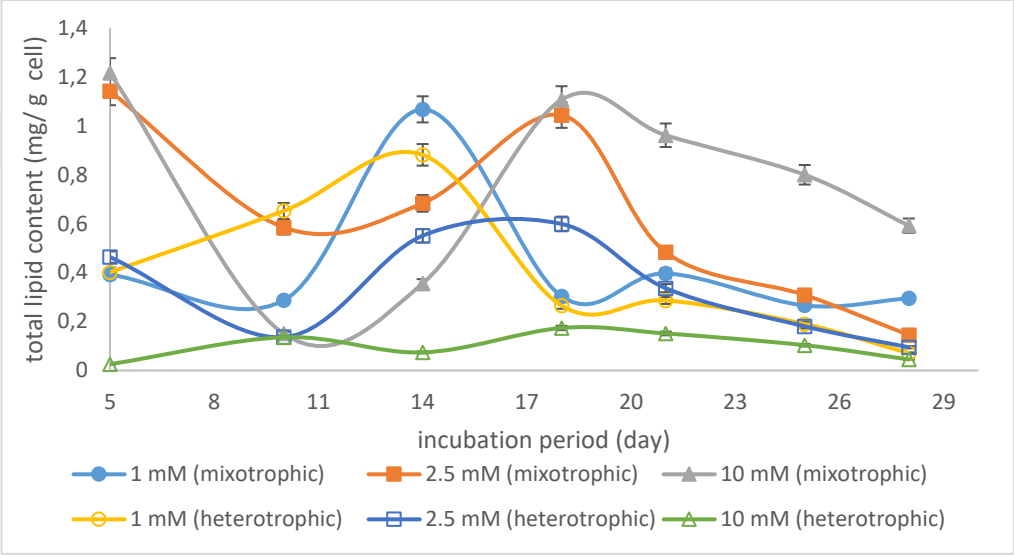

Figure 3.15 Total lipid content changes of *H. pluvialis* cultures in mixotrophic and heterotrophic cultures in the presence of technical glycerol at varying concentrations (1, 2.5 and 10 mM)

3.2.3 Growth of H. pluvialis in the Presence of Whey in Mixotrophic and Heterotrophic Cultures

When the growth curves of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing whey at different concentrations (1, 10 and 30%, v/v) were examined, it was determined that the OD and growth increased with the increase in the waste concentration (Figure 3.16). The maximum OD value (2.37±0.11) was achieved in heterotrophic medium containing 30% (v/v) whey while the maximum specific growth rate (μ= 0.26 day^{-1}) was detected in the mixotrophic culture that contains 30% (v/v) whey ($p<0.05$). In whey containing media, OD values were low at low carbon

source concentrations, while OD values increased at high carbon source concentrations. However, sudden decreases in OD values were observed in the last days of incubation in these media, which may occur because of possible toxic effect of waste materials.

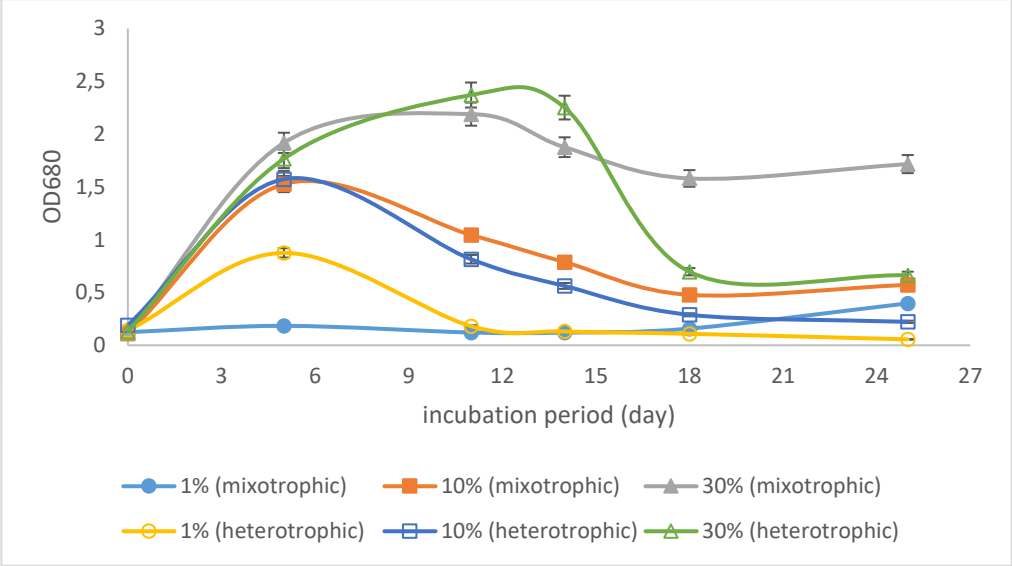

Figure 3.16 OD values of *H. pluvialis* cultures in mixotrophic and heterotrophic cultures in the presence of whey at varying concentrations (1, 10 and 30%, v/v)

When the chlorophyll change was examined during the incubation period, the chlorophyll a values showed a similar trend and increased in the mixotrophic medium containing 1% (v/v) whey in the last days of the incubation (Figure 3.17). The maximum chlorophyll a (41.21±0.18 mg/ g cell) level was detected on the 25th day of incubation in mixotrophic culture including 1% (v/v) whey and the maximum chlorophyll b (56.74±0.26 mg/ g cell) content was detected 10th of incubation in heterotrophic medium containing 1% (v/v) whey ($p<0.05$). The reason for the increase in the amounts of chlorophyll a and b in the last days of incubation in cultures with low concentration of whey can be interpreted as microalgae needs more photosynthesis due to the decline in the carbon source in the medium. The reason why the amount of chlorophyll a is lower than b in the heterotrophic environment is that chlorophyll a degrades in the dark (Maroneze, Zepka, Lopes, Pérez-Gálvez & Roca, 2019). Chlorophyll b structure, on the other hand, is less exposed to degradation because it is less oxidized.

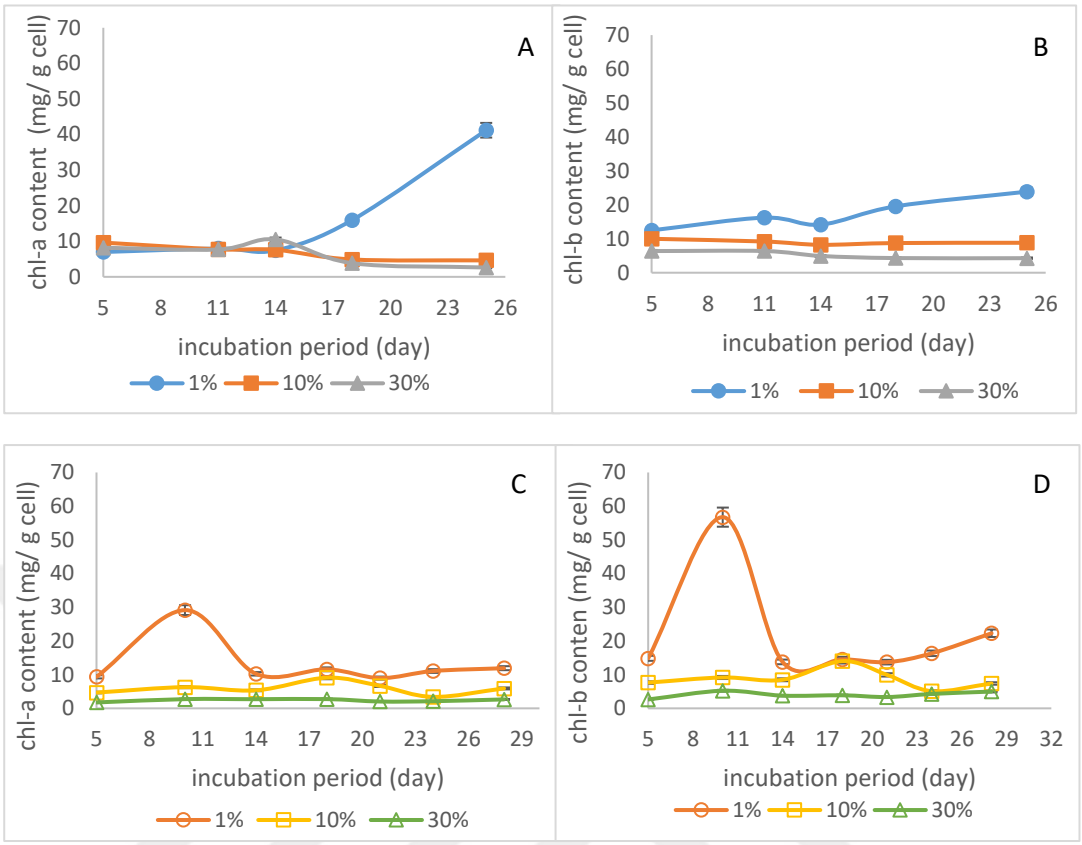

Figure 3.17 Chlorophyll a and chlorophyll b content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v) (A) Chlorophyll a in mixotrophic culture, (B) Chlorophyll b in mixotrophic culture, (D) Chlorophyll b in heterotrophic culture

The maximum protein value (1.13±0.41 mg/ g cell) was detected on the 25th day of incubation in mixotrophic culture including 1% (v/v) whey ($p<0.05$) (Figure 3.18). In media other than the growth medium where the maximum value was determined, protein values showed a decreasing trend during the incubation period. This may indicate that *H. pluvialis* cannot adapt to environments containing high concentrations of whey. When the total carbohydrate change in the growth medium was examined, decreases were detected in all six cultures during the incubation period (Figure 3.19). The maximum carbohydrate level (3.84±0.16 mg/ g cell) was reached on the 18th day of incubation in mixotrophic culture including 30% (v/v) whey ($p<0.05$). Since microalgae transfer lactose in the nutrient medium into the cell, the maximum total carbohydrate content may have been determined in the medium containing whey at high concentration.

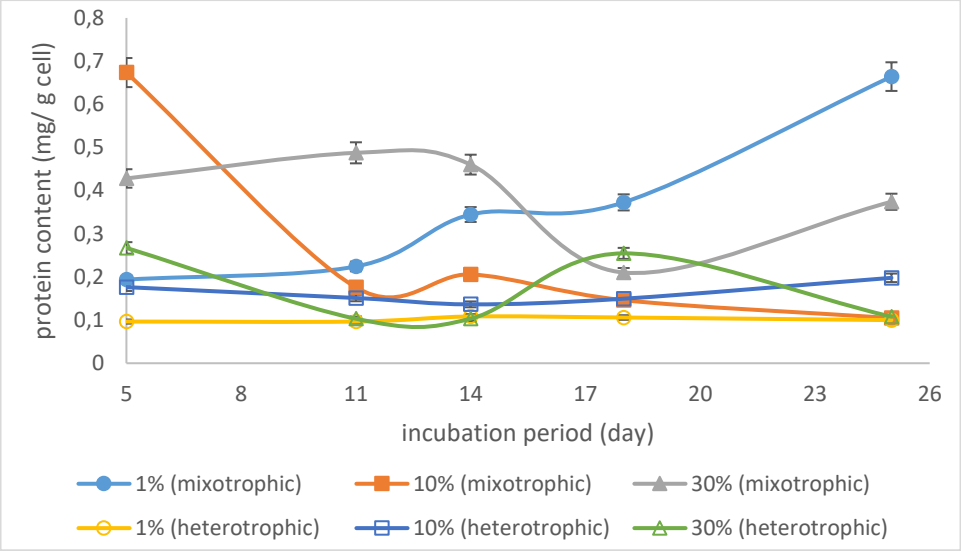

Figure 3.18 Protein content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v)

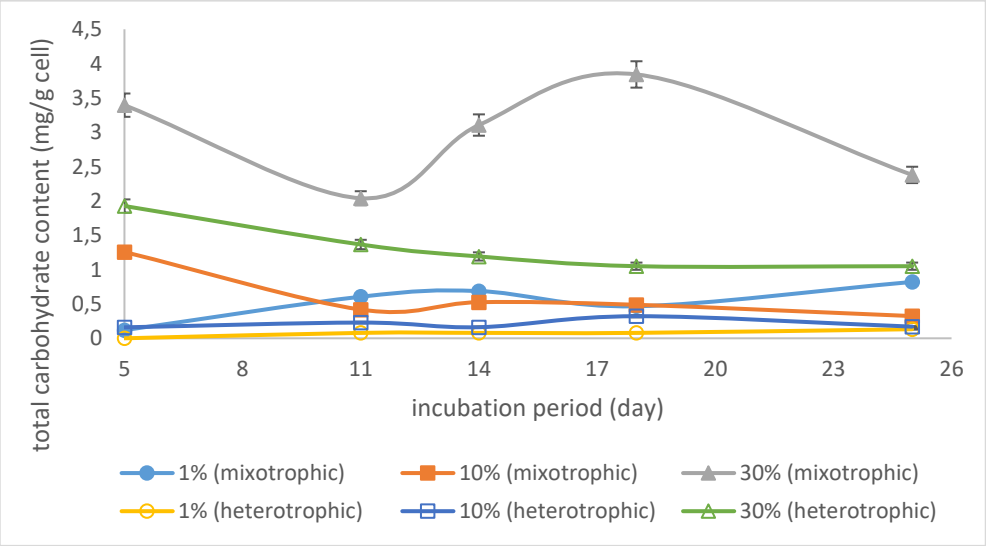

Figure 3.19 Total carbohydrate content changes of *H. pluvialis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v)

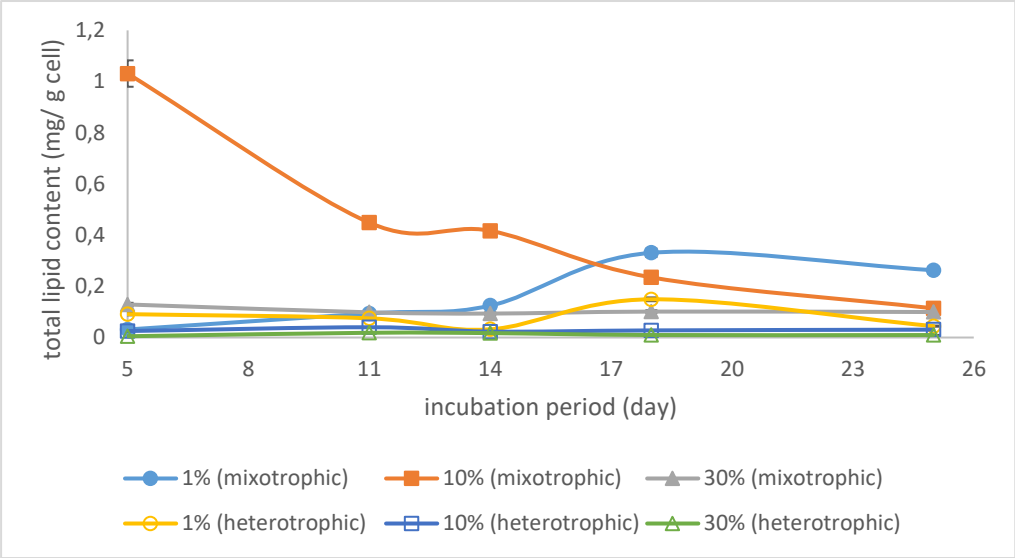

Figure 3.20 Total lipid content changes of *H. pluvialis* cultures in mixotrophic and heterotrophic cultures in the presence of whey at varying concentrations (1, 10 and 30%, v/v)

When the total lipid changes of *H. pluvialis* grown in growth media containing whey was examined during the incubation period, the maximum value (1.03±0.01 mg/ g cell) was determined on the 5th day of incubation in the mixotrophic culture including 10% (v/v) whey and a decrease trend was detected in the incubation period ($p<0.05$) (Figure 3.20). This value is not higher than the lipid value determined in the medium containing crude glycerol. In other media, no significant changes in lipid production were detected ($p>0.05$).

According to the literature review, no study was found in which *H. pluvialis* was grown in a medium containing whey. In this respect, this thesis study is the first example.

When lipid production by *H. pluvialis* is evaluated in different trophic conditions, it is seen that the highest lipid production occurs in mixotrophic medium containing crude glycerol (Table 3.1). In addition, lipid levels produced in heterotrophic culture remained low compared to other cultivations and maximum lipid levels were detected in later days of incubation. For these reasons, studies continued in mixotrophic medium containing 2.5 mM crude glycerol.

Table 3.1 Maximum lipid production conditions for *H. pluvialis* in different trophic cultures

Trophic culture type	**Carbon source type**	**Carbon source concentration**	**Max lipid production (mg/ g cell)**	**Production time (day)**
Photoautotrophic	-	-	0.99±0.04	28
Mixotrophic	**Crude glycerol**	2.5 mM	4.32±0.22	14
	Technical glycerol	10 mM	1.22±0.05	5
	Whey	10%	1.03±0.01	5
Heterotrophic	**Crude glycerol**	2.5 mM	1.05±0.01	18
	Technical glycerol	1 mM	0.88±0.01	14
	Whey	1%	0.15±0.01	18

3.3 Determination of Appropriate Tropic Condition and Carbon Source for *S. platensis*

3.3.1 Growth of S. platensis in the Presence of Crude Glycerol in Mixotrophic and Heterotrophic Cultures

When the growth curves of *S. platensis* grown in mixotrophic and heterotrophic cultures containing crude glycerol in different concentrations (1, 2.5 and 10 mM) were examined, it was determined that the microalgae adapted to mixotrophic cultures (Figure 3.21). It was determined that the growth was very low in heterotrophic culture. The maximum OD value (2.96±0.13) was detected with 1 mM crude glycerol while the maximum specific growth rate (μ= 0.31 day^{-1}) was detected with 10 mM crude glycerol containing mixotrophic medium ($p<0.05$). The specific growth rates in heterotrophic cultures were lower than in mixotrophic cultures. This situation was supported by protein amounts (Figure 3.23). Various studies show that mixotrophic cultivation raises growth more than heterotrophic cultivation (Wang, Zhou, Shao & Liu, 2017). This situation may be related to the fact that there are more energy and

carbon skeletons produced due to the activity of two different metabolisms in mixotrophic cultivations. Protein content increased during the incubation period in the mixotrophic culture while it decreased in the heterotrophic culture.

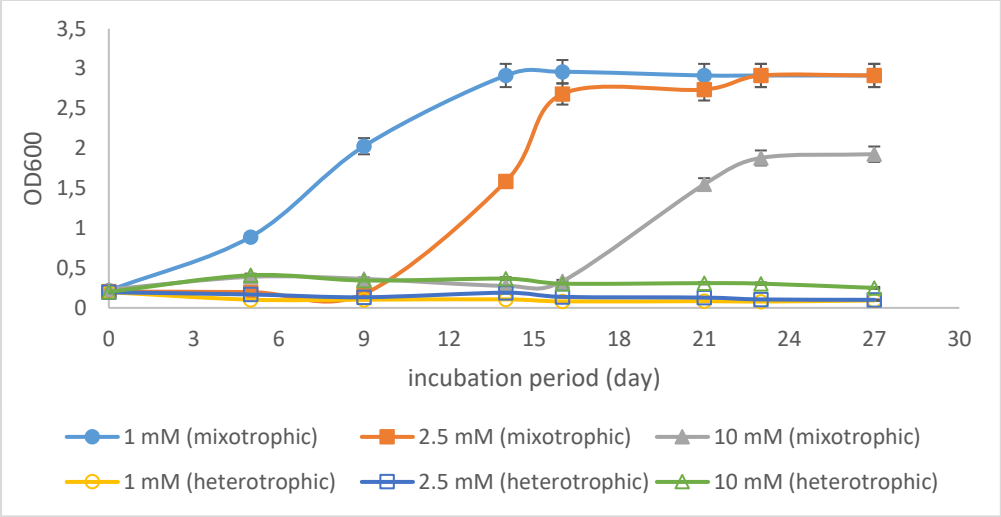

Figure 3.21 OD values of *S. platensis* cultures in mixotrophic and heterotrophic cultures in the presence of crude glycerol at varying concentrations (1, 2.5 and 10 mM)

When the chlorophyll values in these cultures are examined, it is seen that the chlorophyll a values are higher in the mixotrophic cultures while the chlorophyll b values are higher in the heterotrophic cultures (Figure 3.22). The maximum chlorophyll a value (301.35±1.46 mg/ g cell) was determined in mixotrophic culture containing 1 mM crude glycerol on 14th day of incubation ($p<0.05$). This result may have been encountered in cultures with high carbon sources due to the fact that the amount of organic carbon in the media is sufficient and there is less need for photosynthesis. The maximum chlorophyll b value (282.54±1.38 mg/ g cell) was determined in heterotrophic culture containing 2.5 mM crude glycerol on 14th day of incubation ($p<0.05$). With the decrease in the amount of external carbon source, the activity of photoautotrophic metabolism increased and the amount of chlorophyll increased. In the heterotrophic culture, the amount of chlorophyll decreased during the incubation period. This indicates the adaptation process of the inoculum from photoautotrophic culture to heterotrophic culture. The presence of bioactive compounds such as chlorophyll a in the microalgae is relevant to the effectiveness of the photosynthetic activity, it appraises its adaptation to environmental stress status

(Da Silva Vaz, Moreira, de Morais & Costa, 2016). These effects can direct the metabolism to the synthesis of biomolecules.

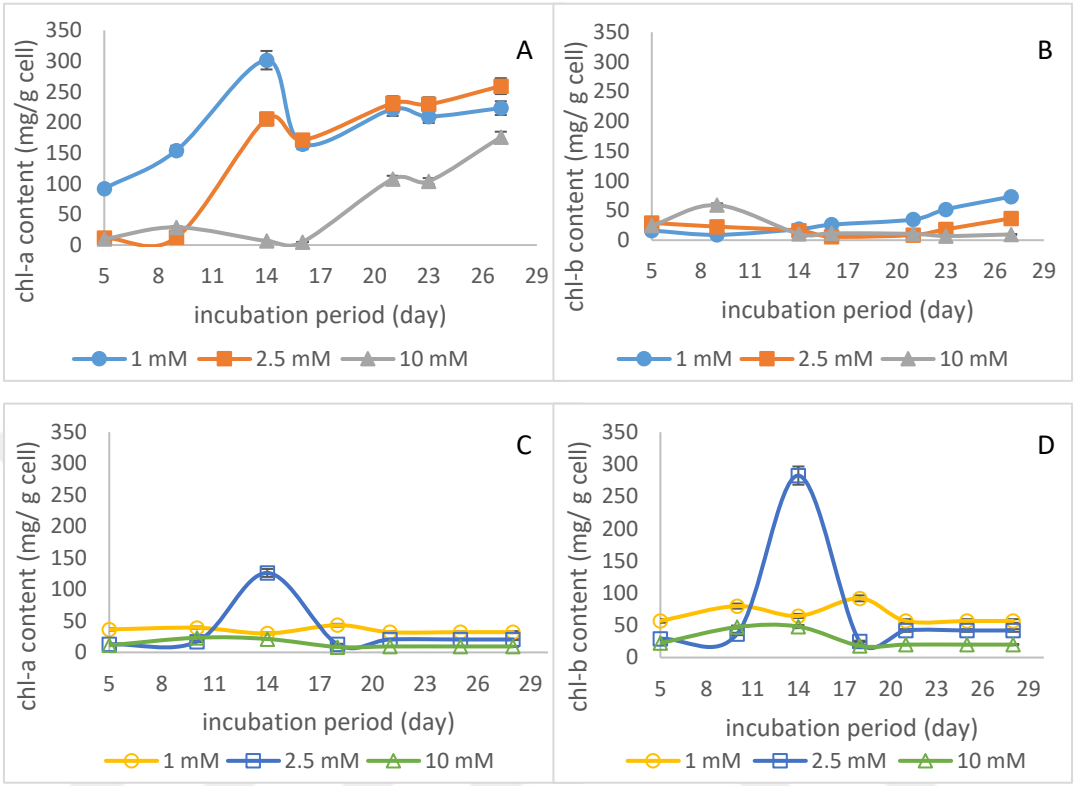

Figure 3.22 Chlorophyll a and chlorophyll b content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing crude glycerol at varying concentrations (1, 2.5 and 10 mM) (A) Chlorophyll a in mixotrophic culture, (B) Chlorophyll b in mixotrophic culture, (C) Chlorophyll a in heterotrophic culture, (D) Chlorophyll b in heterotrophic culture

While high protein values were detected in mixotrophic cultures, protein values were found to be low in heterotrophic cultures (Figure 3.23). The detected protein values are parallel to the OD values. Based on these results, it can be said that *S. platensis* cannot adapt to heterotrophic cultures. The maximum protein content (28.92±0.13 mg/ g cell) was detected in mixotrophic culture containing 1 mM glycerol on 25th day of incubation ($p<0.05$). A decrease was observed in protein values as well as OD values of higher crude glycerol concentrations. It can be said that glycerol concentration above 1 mM is not suitable for biomass production.

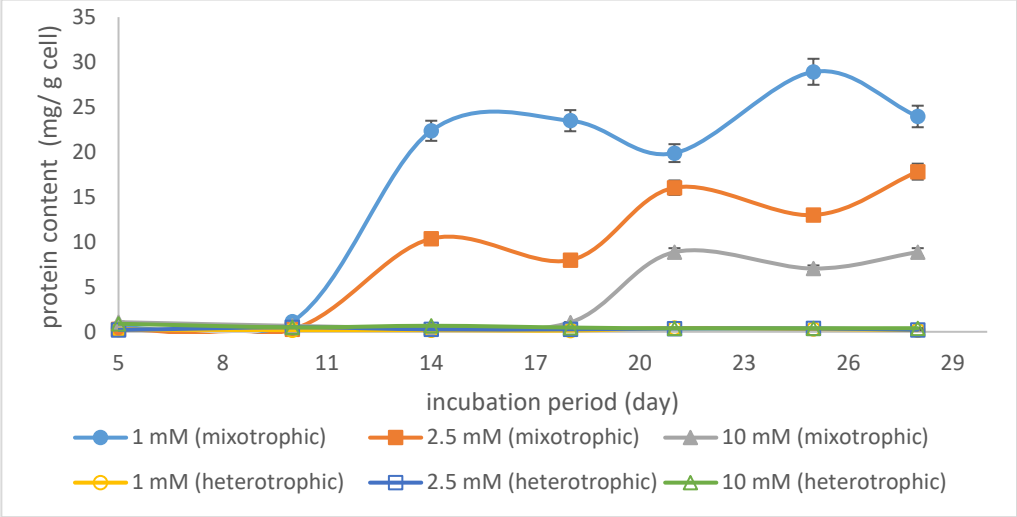

Figure 3.23 Protein content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing crude glycerol at varying concentrations (1, 2.5 and 10 mM)

The low protein value in heterotrophic culture parallels the low specific growth rate (Figure 3.21). While protein and chlorophyll a levels were high in the first 17 days in the mixotrophic medium, they increased after this day in the heterotrophic medium. In mixotrophic culture, low chlorophyll values in the first days of incubation indicate that heterotrophic metabolism is active (Figure 3.22). Glycerol, which is used as an external carbon source, can be easily transferred into the cell and metabolized and provides a carbon skeleton while supporting the acetyl-CoA pool for lipid production. For this reason, glycerol was used in the first days when lipid production increased, and in the following days, photoautotrophic metabolism may have been activated. The fixed carbon dioxide by chlorophyll, provides a carbon source that can be used in lipid production in addition to the organic carbon source (Goncalves et al., 2016). In our study, crude glycerol, as an external carbon source, was consumed approximately on the second day of incubation, and this result is similar to the study of De Morais et al. (2020).

Total carbohydrate values were in a general increasing trend (Figure 3.24). There was no significant change in carbohydrate content among growth media ($p>0.05$). The maximum total carbohydrate content (0.47±0.02 mg/ g cell) was detected in mixotrophic culture containing 1 mM glycerol on 21st day of incubation ($p<0.05$). This shows that the assimilated organic carbon source and fixed CO_2 were used in lipid and

protein production. In a study, it was determined that carbohydrate production decreased while protein and lipid production increased in glycerol containing medium (Morais et al., 2019). Markou et al. (2019) suggest that glycerol has an energy carrier function in proteins and lipids synthesis, apart from carbohydrates in the *S. platensis* culture. This molecule has an energy carrier function due to the carbon skeleton and chemical bond energy it contains.

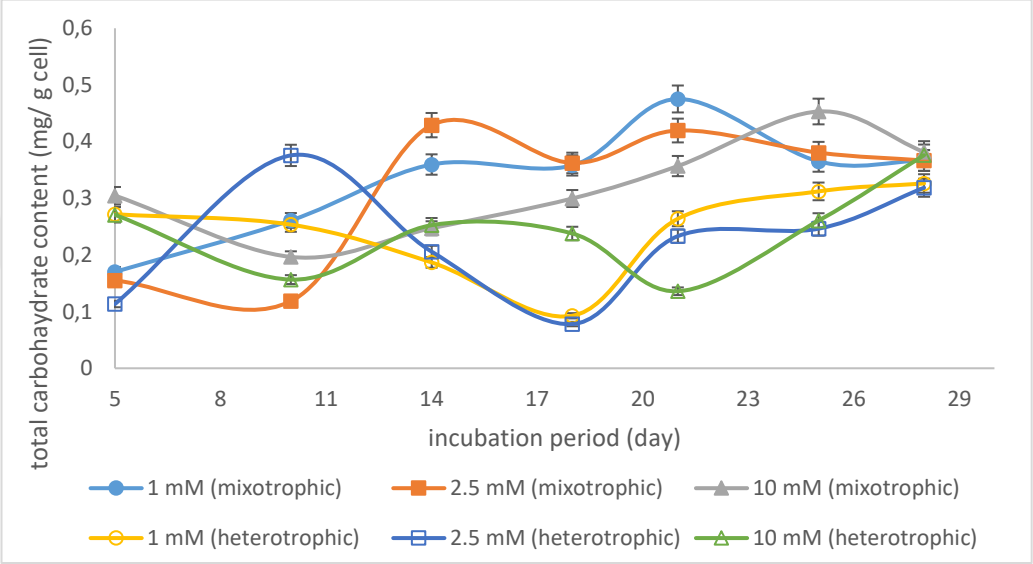

Figure 3.24 Total carbohydrate content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing crude glycerol at varying concentrations (1, 2.5 and 10 mM)

When the total lipid changes during the incubation period of *S. platensis* grown in growth media containing crude glycerol was examined the maximum lipid content (5.78±0.21 mg/g cell) was detected in mixotrophic culture containing 10 mM crude glycerol ($p<0.05$) (Figure 3.25). Approximately 19% more production was achieved than the amount of lipid produced in photoautotrophic cultivation. Due to both metabolisms work together, lipid generation is extra effective in mixotrophic culture according to the other trophic types in which only photoautotrophic or heterotrophic metabolisms carry out (Kong et al., 2013). In mixotrophic cultures it is an advantage to assimilating organic substrates and having active photosynthetic pathway because acetyl-CoA pool is supported by CO_2 fixation and extracellular carbon. The cell is not dependent only on light or only on the organic carbon source. Both photoautotrophic and heterotrophic metabolisms produce energy and provide carbon skeleton for lipid

production. It is obvious that the extravagantly produced energy is stored in TAG format. However, in heterotrophic conditions organic carbon source is the only carbon and energy source that the cyanobacteria could use. For this reason, the amount of lipid and protein remained low in the heterotrophic cultivation, as the available carbon is used both in the growth of the cell and in the production of the necessary storage material.

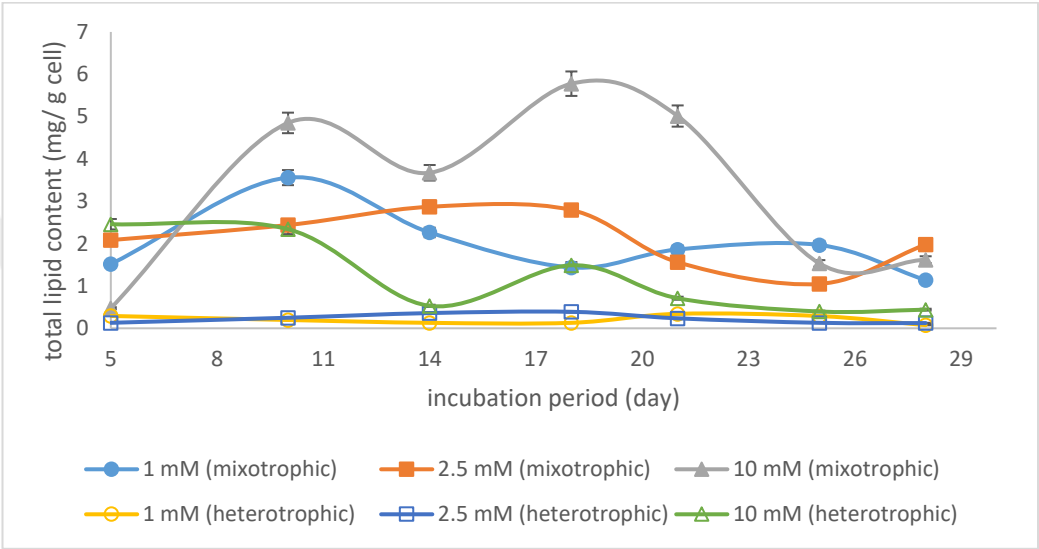

Figure 3.25 Total lipid content changes of *S. platensis* cultures in mixotrophic and heterotrophic cultures in the presence of crude glycerol at varying concentrations (1, 2.5 and 10 mM)

In this present study higher glycerol conditions provide higher C/N ratio for both production medium, while there is no nitrogen in the content of crude glycerol, carbon content is high. Due to there is not enough N for protein synthesis in the medium, the fixed carbon is used in the synthesis of storage material such as TAG. This mechanism comes to the forefront especially in mixotrophic production.

3.2.2 Growth of S. platensis in the Presence of Technical Glycerol in Mixotrophic and Heterotrophic Cultures

When the growth curves of *S. platensis* grown in mixotrophic or heterotrophic cultures containing technical glycerol in different concentrations (1, 2.5 and 10 mM) are examined it was determined that microalgae grew better in mixotrophic cultures, but did not grow in heterotrophic cultures (Figure 3.26). The maximum specific growth

rate (μ= 0.13 day^{-1}) was detected in 10 mM technical glycerol containing mixotrophic medium and high OD values were also detected in mixotrophic cultures ($p<0.05$).

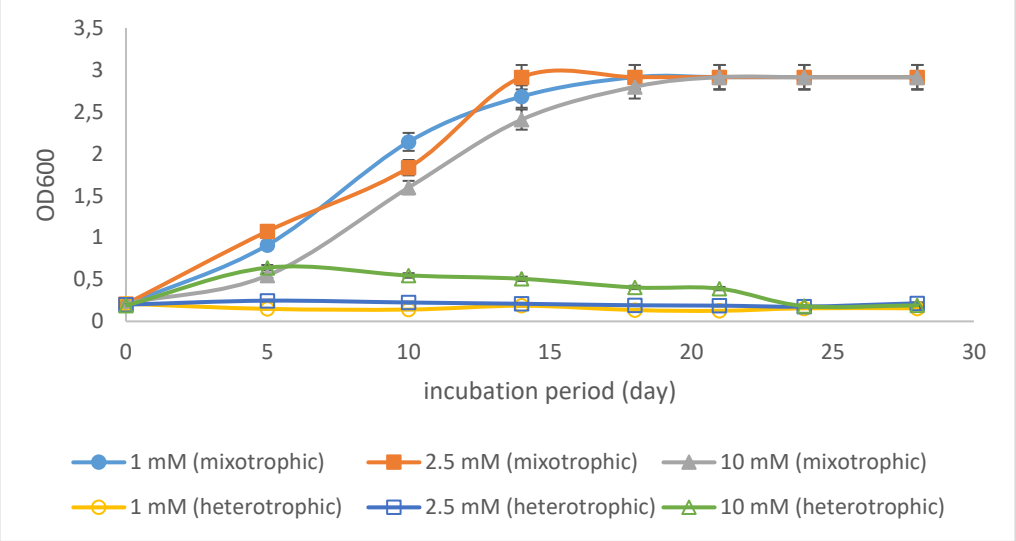

Figure 3.26 OD values of *S. platensis* cultures in mixotrophic and heterotrophic cultures in the presence of technical glycerol at varying concentrations (1, 2.5 and 10 mM)

Chlorophyll a content increased during the incubation period in mixotrophic medium (Figure 3.27). This situation can be interpreted as heterotrophic assimilation metabolism is more active in the first days of incubation. It has been determined that chlorophyll levels are lower in heterotrophic media. The maximum chlorophyll a level (333.53±1.48 mg/ g cell) was detected in mixotrophic medium containing 1 mM technical glycerol on 27^{th} day of incubation, while the maximum chlorophyll b content (119.59±0.6 mg/ g cell) was determined in heterotrophic medium containing 2.5 mM technical glycerol on 18^{th} day of incubation ($p<0.05$). It is thought to be due to the fact that the chlorophyll degradation rate in the dark environment is different for chlorophyll a and b (Maroneze et al., 2019).

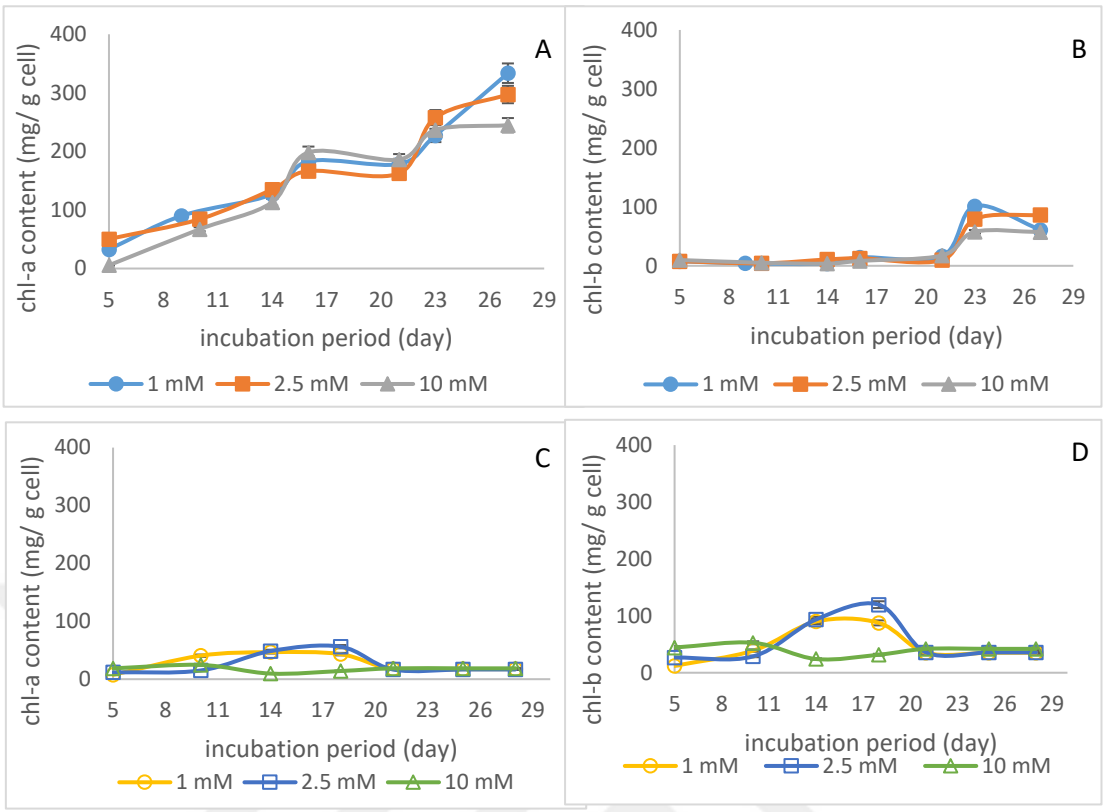

Figure 3.27 Chlorophyll a and chlorophyll b content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing technical glycerol at varying concentrations (1, 2.5 and 10 mM) (A) Chlorophyll a in mixotrophic culture, (B) Chlorophyll b in mixotrophic culture, (C) Chlorophyll a in heterotrophic culture, (D) Chlorophyll b in heterotrophic culture

While high protein values were detected in mixotrophic media, protein values remained low in heterotrophic media (Figure 3.28). These results were also supported by the OD values. The maximum protein level (38.67±0.17mg/ g cell) was determined in mixotrophic culture containing 2.5 mM technical glycerol on 28th day of incubation ($p<0.05$).

Total carbohydrate values did not change significantly during incubation in heterotrophic and mixotrophic media and high values were not reached ($p>0.05$) (Figure 3.29). This may indicate that the microalgae in these media do not prefer carbohydrate as a storage material. Instead, it may have preferred biomass increase and lipid production. Protein and lipid contents also support this assumption.

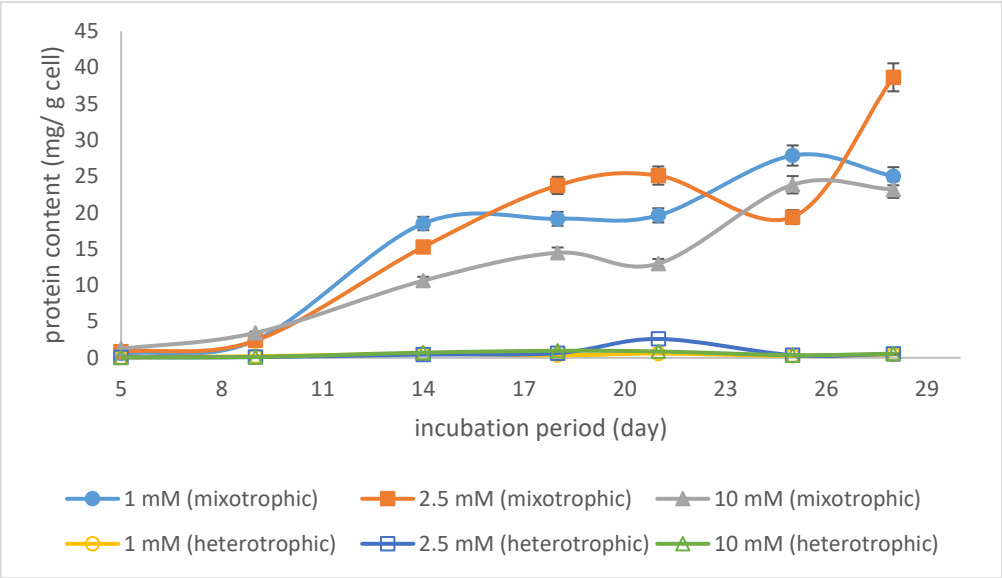

Figure 3.28 Protein content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing technical glycerol at varying concentrations (1, 2.5 and 10 mM)

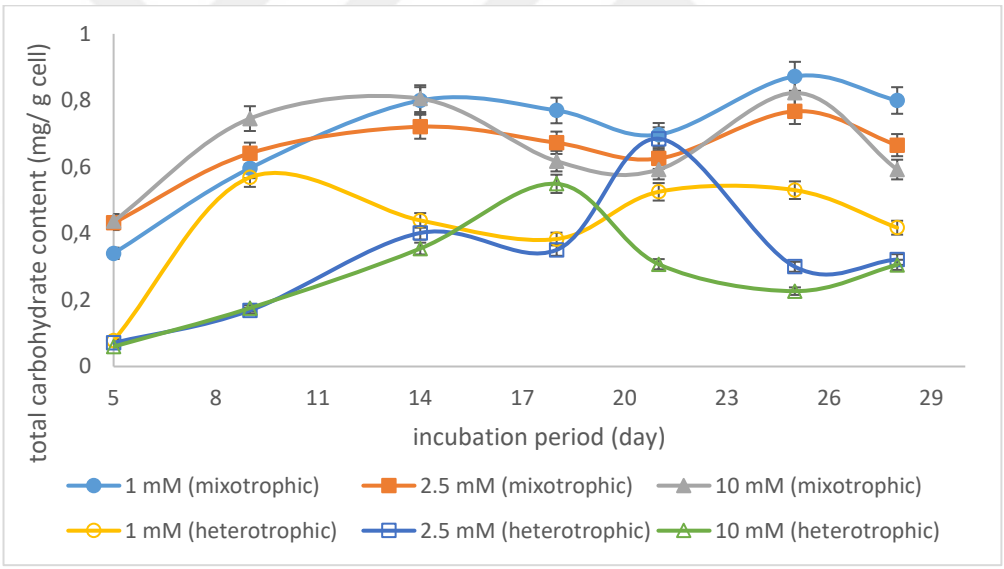

Figure 3.29 Total carbohydrate content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing technical glycerol at varying concentrations (1, 2.5 and 10 mM)

When the total lipid changes of *S. platensis* grown in technical glycerol-containing growth media was examined during the incubation period, high values were reached in mixotrophic cultures (Figure 3.30). The maximum value (2.62±0.12 mg/ g cell) was detected in mixotrophic culture containing 1 mM technical glycerol on the 14th day of incubation ($p<0.05$). However, this value is lower than the lipid produced in the control condition.

A study in which *Spirulina* sp. was grown at a glycerol concentration varying between 10-50 mM, it was determined that while the glycerol concentration increased, protein and biomass production increased, total carbohydrate content decreased, and lipid content did not show a significant change (De Morais, Druzian, Nunes, de Morais & Costa, 2019).

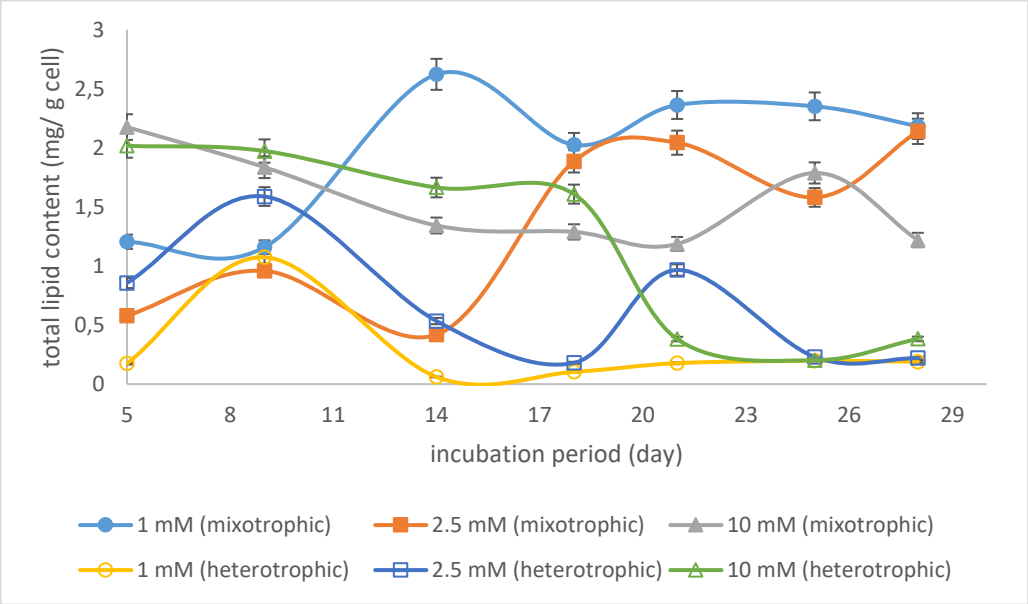

Figure 3.30 Total lipid content changes of *S. platensis* cultures in mixotrophic and heterotrophic cultures in the presence of technical glycerol at varying concentrations (1, 2.5 and 10 mM)

3.2.3 Growth of S. platensis in the Presence of Whey in Mixotrophic and Heterotrophic Cultures

When the growth curves of *S. platensis* grown in mixotrophic and heterotrophic cultures containing whey in different concentrations (1, 10 and 30%, v/v) were examined, it was determined that the microalgae adapted to all six cultures (Figure 3.31). The maximum OD value (2.74±0.12) was detected in mixotrophic medium containing 1% (v/v) whey and the maximum specific growth rate (μ= 0.2 day^{-1}) was detected in heterotrophic medium containing 30% (v/v) whey ($p<0.05$). Mixotrophic cultivation more promotes growth according to heterotrophic cultivation (Wang et al., 2017; Zhan et al., 2017). However, the heterotrophic medium with higher concentration of whey may have created the favorable condition for the growth of the

cyanobacteria, resulting in higher specific growth rate. In the mixotrophic culture including high whey, high OD and specific growth rates may not have been determined, since the necessary order for the simultaneous operation of the two metabolisms could not be established. In mixotrophic cultivation, cells require a lower organic carbon source because higher carbon source concentration can have an inhibition effect (Joannes, Mansa, Yasir & Dayou, 2016).

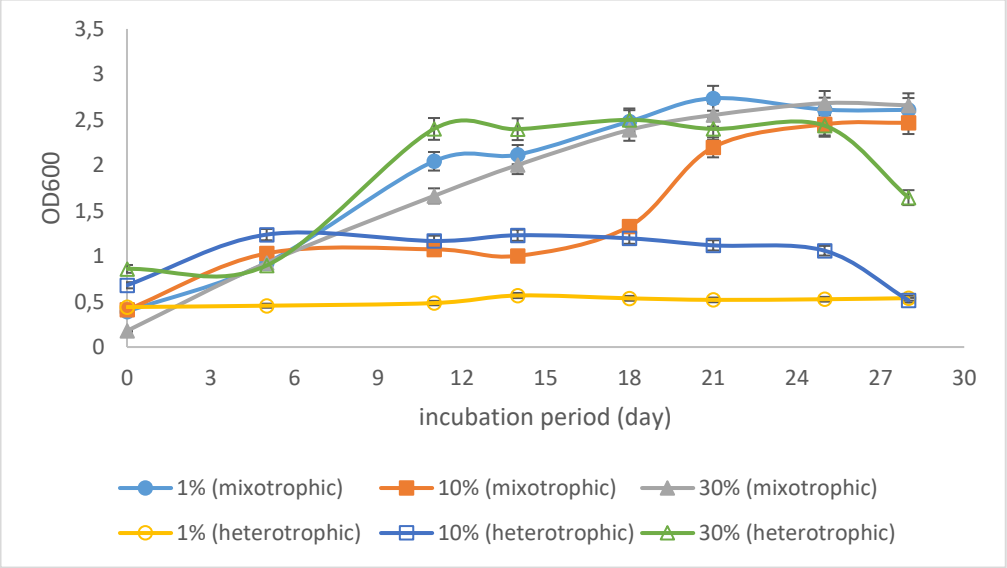

Figure 3.31 OD values of *S. platensis* cultures in mixotrophic and heterotrophic cultures in the presence of whey at varying concentrations (1, 10 and 30%, v/v)

When chlorophyll change was examined during the incubation period, chlorophyll a values increased in the last days of incubation in mixotrophic cultures containing 1% (v/v) and 10% (v/v) whey, but did not show a significant change in the medium containing 30% (v/v) whey (Figure 3.32). The maximum chlorophyll a (292.39±1.31 mg/ g cell) was detected on the 28th day in the culture including 10% (v/v) whey, and the chlorophyll b (67.59±0.31 mg/ g cell) value was detected on the 21st day of the incubation in the heterotrophic culture including 1% (v/v) whey ($p<0.05$). The reason for the increase in the amount of chlorophyll in the mixotrophic medium containing 10% (v/v) whey in the last days of incubation is that it used the organic carbon source in the medium in the first days of incubation and then activated its photoautotrophic metabolism. This is also supported by the total carbohydrate content data (Figure 3.34). As expected, chlorophyll levels were detected at higher values in mixotrophic

culture. Additionally to the organic carbon source, CO_2 supplies a carbon source that can be used in biochemical components production in mixotrophic cultures (Zhu et al., 2016). The low amount of chlorophyll in heterotrophic cultivation indicates that the cell is adapted to this type of cultivation and that only the heterotrophic metabolism is active.

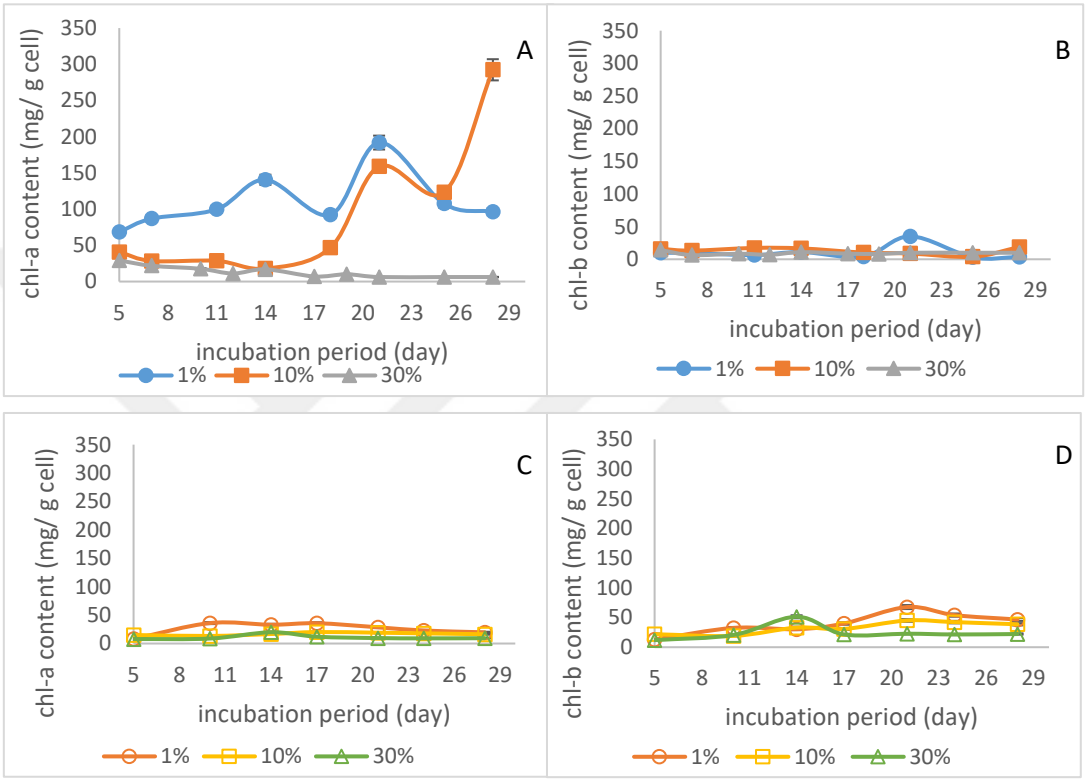

Figure 3.32 Chlorophyll a and chlorophyll b content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v) (A) Chlorophyll a in mixotrophic culture, (B) Chlorophyll b in mixotrophic culture, (C) Chlorophyll a in heterotrophic culture, (D) Chlorophyll b in heterotrophic culture

While the amount of protein did not change significantly during the incubation in mixotrophic media containing 10% (v/v) and 30% (v/v) whey an increase was observed in the medium containing 1% (v/v) whey (Figure 3.33). This result is also supported by the OD data. In the mixotrophic cultures, the protein content has been found to be very low. The maximum protein level (3.76±0.14 mg/ g cell) was determined in heterotrophic culture that contains 1% (v/v) whey (Figure 3.33) ($p<0.05$). The maximum protein contest of whey might have caused this result.

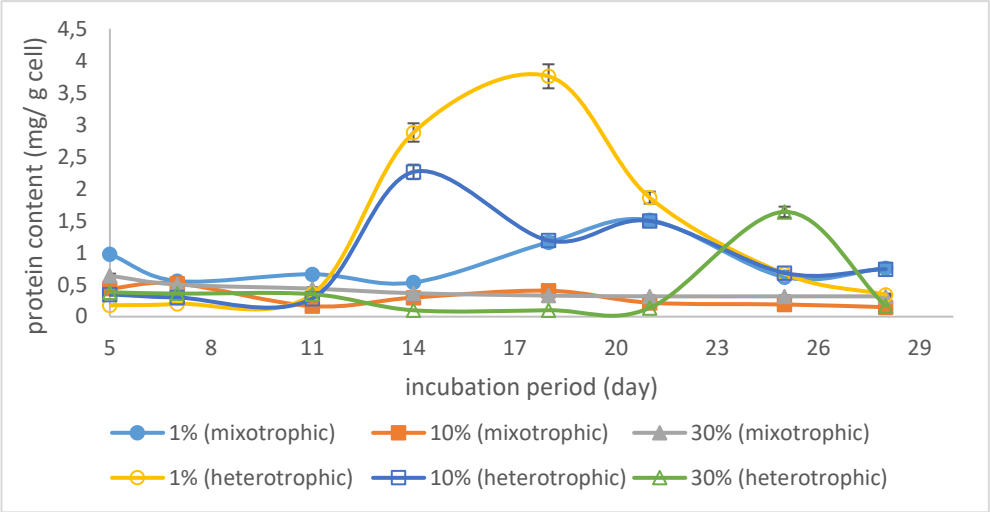

Figure 3.33 Protein content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v)

When the total carbohydrate change in the growth medium was examined, it was determined that it showed an increasing trend during the incubation period (Figure 3.34). The maximum value (1.42±0.67 mg/ g cell) was detected on 21st day of incubation in a mixotrophic culture including 10% (v/v) whey ($p<0.05$). While the carbohydrate contents remained low values the protein level arrived higher levels in the whey containing heterotrophic medium. The presence of both organic and inorganic carbon sources in the mixotrophic culture caused both metabolisms to work. For this reason, the total carbohydrate amount was determined at higher levels than in the heterotrophic medium. However, 30% (v/v) whey creates a high carbon concentration for the mixotrophic medium. It was determined by the chlorophyll, protein and total carbohydrate values that the cells could not adapt to this medium.

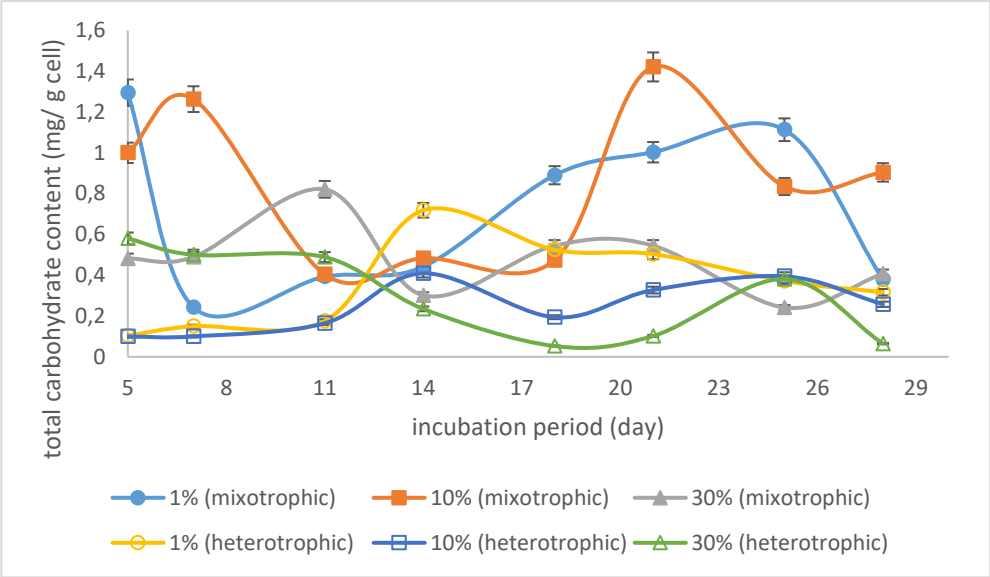

Figure 3.34 Total carbohydrate content changes of *S. platensis* grown in mixotrophic and heterotrophic cultures containing whey at varying concentrations (1, 10 and 30%, v/v)

When the total lipid changes of *S. platensis* grown in growth media containing whey was examined during the incubation period, it was determined that it showed a similar trend in mixotrophic cultures and heterotrophic cultures (Figure 3.35). The maximum total lipid value (4.67±0.18 mg/ g cell) was reached on the 18th day of incubation in heterotrophic culture including 1% (v/v) whey ($p<0.05$). There was no meaningful difference according to the amount of lipid produced in the control condition. The maximum lipid level was detected in the last days of production process in which contains higher whey concentrations. In mixotrophic cultivations the maximum lipid production was determined as 3.76±0.16 mg/ g cell in which contains 10% (v/v) whey on the 14th day of production process. The maximum lipid values were obtained at the beginning of the stationary phase.

In a similar study, *S. platensis* was grown in mixotrophic medium containing varying concentrations of whey (Pereira et al., 2019). As in this thesis study, the maximum specific growth rate was determined in the medium containing the highest organic carbon (10%). In addition, it was determined that the lipid content decreased in mixotrophic cultures compared to the control condition (photoautotrophic culture).

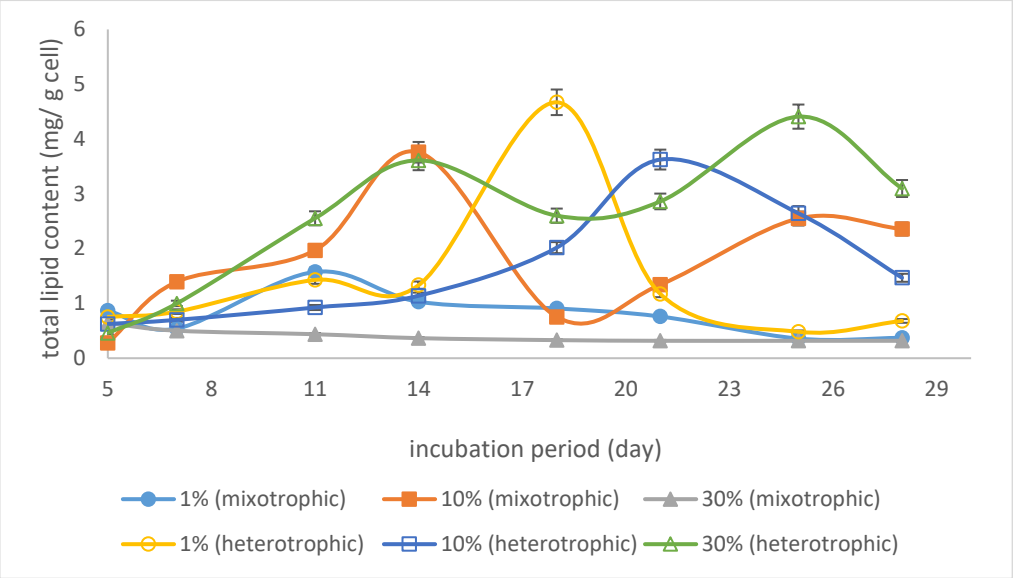

Figure 3.35 Total lipid content changes of *S. platensis* cultures in mixotrophic and heterotrophic cultures in the presence of whey at varying concentrations (1, 10 and 30%, v/v)

Table 3.2 Maximum lipid production conditions for *S. platensis* in different trophic cultures

Trophic culture type	**Carbon source type**	**Carbon source concentration**	**Max lipid production (mg/ g cell)**	**Production time (day)**
Photoautotrophic	-	-	4.58±0.22	3
Mixotrophic	**Crude glycerol**	10 mM	5.78±0.21	18
	Technical glycerol	1 mM	2.62±0.12	14
	Whey	10%	3.76±0.17	14
Heterotrophic	**Crude glycerol**	10 mM	2.46±0.11	5
	Technical glycerol	10 mM	2.02±0.09	5
	Whey	1%	4.67±0.18	18

When lipid production by *S. platensis* is evaluated in different trophic conditions, it is seen that the highest lipid production occurs in mixotrophic medium containing crude glycerol (Table 3.2). However, considerable lipid production has also occurred

in heterotrophic cultures, but lower lipid was produced compared to photoautotrophic and mixotrophic cultures. For these reasons, studies continued in mixotrophic medium containing 10 mM crude glycerol.

3.3 Determination of Nutritional and Environmental Parameters Effects on Lipid Production

After determining the suitable cultivation type and carbon source and concentration for *H. pluvialis* and *S. platensis*, the effect of nitrogen concentration, light intensity and phosphorus concentration on lipid production were investigated.

3.3.1 Effects of Nitrogen Concentration on H. pluvialis Culture

The highest lipid content was detected in the mixotrophic culture of *H. platensis* presence of 2.5 mM crude glycerol. When the changes in protein, total carbohydrate and total lipid levels depending on the incubation period are examined in the *H. platensis* cultures where the highest total lipid value is determined, it is seen that all three metabolite levels first increase and then decrease and remain at a certain level (Figure 3.8-3.9-3.10). It can be said that *H. pluvialis* grown in a mixotrophic culture tries to adapt to the medium in the early stages of incubation and photoautotrophic metabolism is active. In photosynthetic metabolism, CO_2 is converted into glycerate-3-phosphate to synthesize storage material compounds such as carbohydrates and lipids. The lipid biosynthesis pathway begins with the conversion of glycerate-3-phosphate to pyruvate and then acetyl CoA. The results obtained showed that in the first days of incubation, *H. pluvialis* increased the carbohydrate and lipid storage by activating the photoautotrophic metabolism, and in the following days, the content of the storage material increased with the increase of heterotrophic metabolism and remained at a certain value. In the next step after these conditions were determined, the effect of nitrogen concentration in the growth medium was investigated.

When the growth of *H. pluvialis* in terms of OD is examined in media containing nitrogen at different concentrations (0, 1.5 and 2.9 mM $NaNO_3$), it is seen that the maximum density is reached in the medium containing 1.5 mM nitrogen (Figure 3.36).

In addition, a higher specific growth rate (0.03 day^{-1}) was determined in the same medium compared to the other two media ($p<0.05$). It has been determined that in the medium where there is no nitrogen, the microalgae cannot adapt to the environment and almost never grow. Considering that nitrogen participates in the structure of proteins, which are the building blocks of cells, it is expected that biomass production will remain low at low nitrogen content.

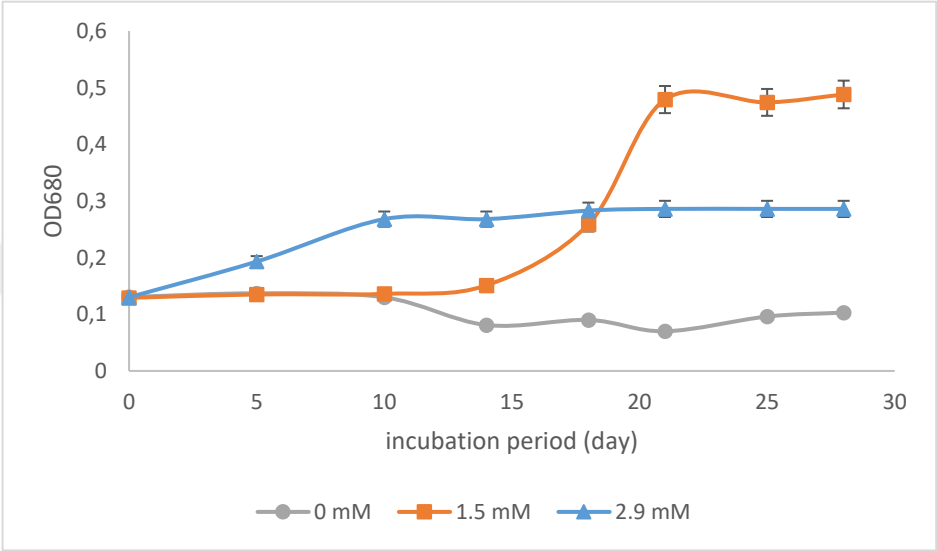

Figure 3.36 OD values of *H. pluvialis* in mixotrophic culture containing 2.5 mM crude glycerol with different nitrogen concentrations (0, 1.5 and 2.9 mM)

It has been determined that chlorophyll a and b levels in the same medium follow a similar trend (Figure 3.7A and B). The increase in the levels of both carotenoid species in the first days of incubation can be interpreted as the first photoautotrophic metabolism of the microalgae. In this process, it is thought to try to adapt to the mixotrophic culture. In addition, the increase in intracellular total carbohydrate and total lipid levels at this stage indicates that it accumulates storage metabolites. The decrease in chlorophyll levels in the later days of the incubation can be interpreted as an indication that *H. pluvialis* has adapted to the mixotrophic culture and assimilated crude glycerol, which is present as a ready-made carbon source, into the cell, thus reducing its photoautotrophic metabolism and increasing its heterotrophic metabolism.

It was determined that chlorophyll a and b levels decreased with the decrease of nitrogen concentration in the production media (Figure 3.37). The maximum chlorophyll a (74.53±0.33 mg/ g cell) and b (50.20±0.22 mg/ g cell) levels were reached in the medium containing 2.9 mM nitrogen ($p<0.05$). Since nitrogen is an important element in the chlorophyll structure, it is normal for the amount of chlorophyll to decrease at low nitrogen concentration. In microalgae grown in low nitrogen containing cultures, degradation of phycobilisomes, which are the light-collecting antennae of photosystem II, occurs; chlorophyll synthesis and photosynthesis rate decreases (Juneja et al., 2013).

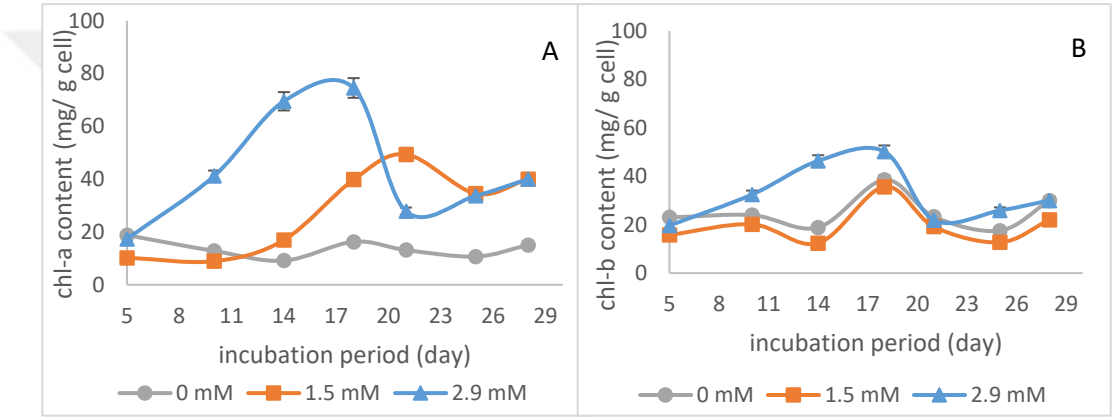

Figure 3.37 Chlorophyll a and chlorophyll b content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different nitrogen concentrations (0,1.5 and 2.9 mM) (A) Chlorophyll a, (B) Chlorophyll b

Protein content is also expected to be high at high nitrogen concentrations. In line with this expectation, in the results showing the protein content, the protein content increased with the increase in nitrogen concentration (Figure 3.38). The maximum protein level was detected as 2.88±0.13 mg/ g cell in the presence of 2.9 mM nitrogen ($p<0.05$) (Figure 3.38). In parallel with the protein content, the total carbohydrate content within the cell also increases with increasing nitrogen concentration (Figure 3.39). The maximum total carbohydrate level was detected as 0.28±0.01 mg/ g cell in the presence of 2.9 mM nitrogen ($p<0.05$). This result shows that the cell grows more in the medium containing 2.9 mM nitrogen and can take more carbohydrates.

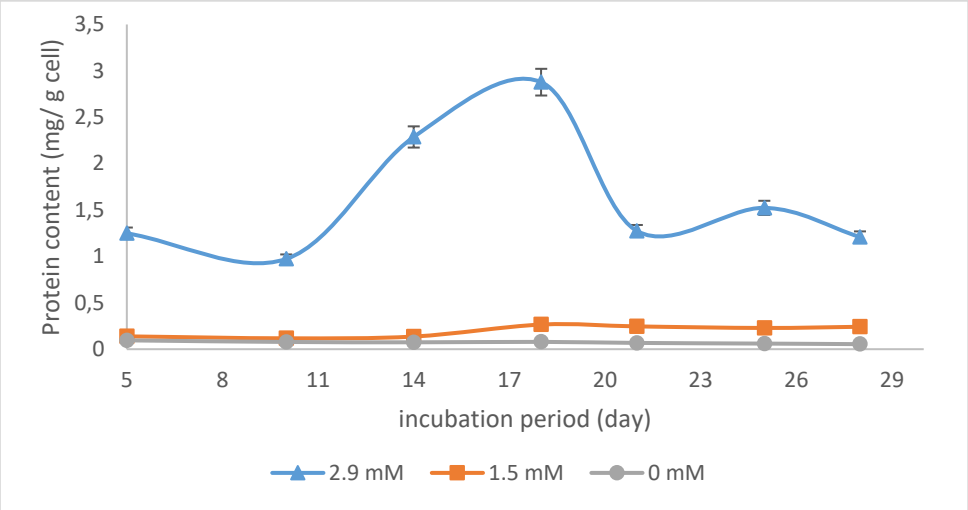

Figure 3.38 Protein content changes of *H. pluvialis* grown in cultures containing 2.5 mM crude glycerol with different nitrogen concentration (0, 1.5 and 2.9 mM)

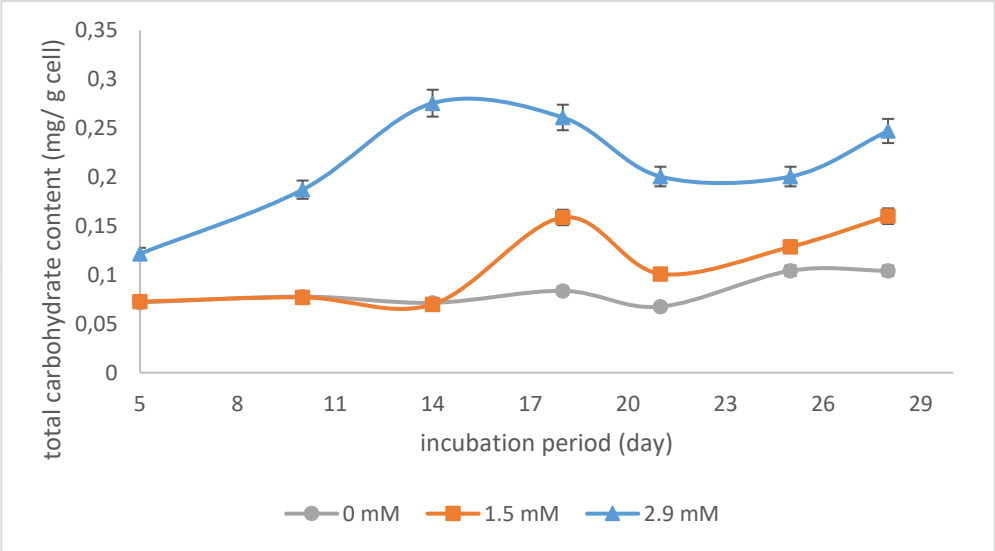

Figure 3.39 Total carbohydrate content changes of *H. pluvialis* grown in cultures containing 2.5 mM crude glycerol with different nitrogen concentration (0, 1.5 and 2.9 mM)

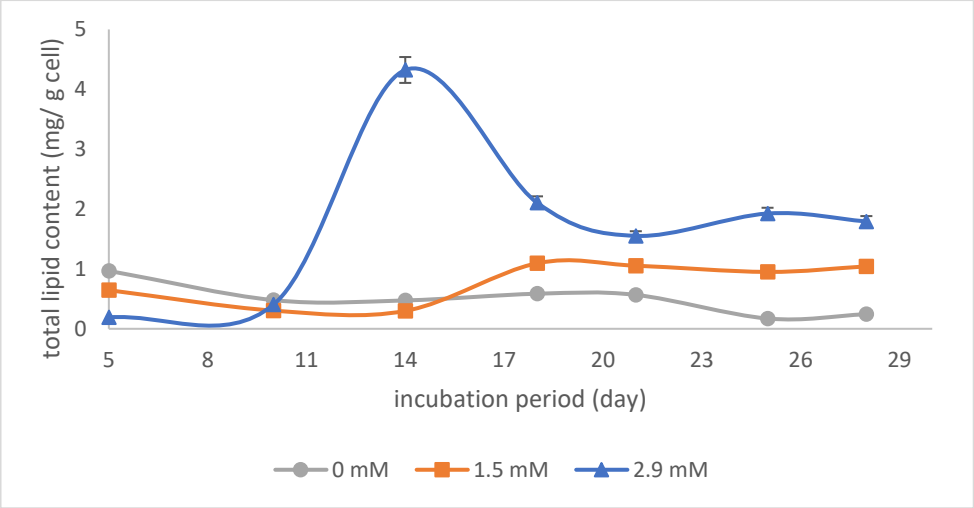

Figure 3.40 Lipid content changes of *H. pluvialis* grown in cultures containing 2.5 mM crude glycerol with different nitrogen concentration (0, 1.5 and 2.9 mM)

The effect of the change in nitrogen concentration in the production medium on the total lipid level was investigated (Figure 3.40). In the absence and scarcity of nitrogen, lipid content remained constant in the first days of incubation, but increased in the following days. The maximum lipid production was detected as 4.32±0.22 mg /g cells with 2.9 mM nitrogen concentration on 14th day of incubation ($p<0.05$). When the data were analyzed by Tukey's HSD test, no significant difference was detected between nitrogen concentration, lipid production, lipid and carbohydrate content ($p>0.05$). There was a moderate positive correlation between protein and lipid production ($r=0.6471$).

In the similar way a study by Morais et al. (2019) showed that the highest lipid productivity occurred with 50 mM glycerol and nitrogen as basal level containing medium. With lower glycerol and nitrogen concentrations lipid productivities decreased because of lower C/N ratio.

3.3.2 Effects of Nitrogen Concentration on S. platensis Culture

The highest lipid production was determined in mixotrophic culture of *S. platensis* in the presence of 10 mM crude glycerol ($p<0.05$). *S. platensis* has produced lipid by adapting to the mixotrophic medium containing crude glycerol. It can be thought that

the heterotrophic assimilation mechanism is active in the first days of incubation. It was determined that OD values (Figure 3.21) and chlorophyll values (Figure 3.22) increased in the last days of incubation in this medium. The low protein value of the cell in this medium may be due to the slowdown in cell division. The high amount of lipids despite the low carbohydrate value can be considered as an indicator that the cell stores excess carbon and energy as lipid. After determining the suitable cultivation type and carbon source for *S. platensis*, the effect of nitrogen concentrations (0, 15 and 29 mM) on lipid production was investigated.

It was determined that microalgae could not adapt to the medium and almost never grow in media where nitrogen was not present and at a concentration of 15 mM nitrogen, while the maximum OD and specific growth rate (μ= 0.31 day^{-1}) were reached in the medium containing 29 mM nitrogen ($p<0.05$) (Figure 3.41). Since nitrogen is an important element in protein and nucleic acid structure, a decrease in cell growth is expected at low nitrogen concentrations.

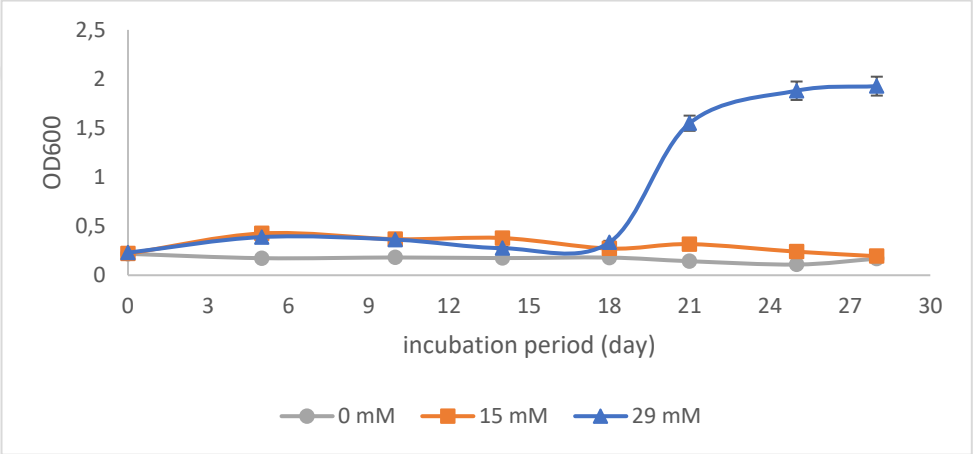

Figure 3.41 OD values of *S. platensis* in mixotrophic culture containing 10 mM crude glycerol with different nitrogen concentrations (0, 15 and 29 mM)

It was determined that the maximum chlorophyll a (175.98±0.86 mg/ g cell) and b (58.96±0.26 mg/ g cell) values were reached in the medium containing 29 mM nitrogen ($p<0.05$) (Figure 3.42). In media with lower nitrogen, both chlorophyll levels remained low. In the medium with the highest nitrogen concentration, it was determined that the amount of chlorophyll b was high in the first days of the

incubation, and the amount of chlorophyll a increased in the following days. In a study conducted with a green algae species, it was determined that chlorophyll synthesis decreased in the presence of low concentrations of nitrogen in the growth medium (Ellis, Spooner & Yakulis, 1975). It was determined that chlorophyll synthesis slowed down especially in media with high C/N ratio, as in this study. This shows that in the first days of incubation, *S. platensis* uses the external carbon source in the medium and therefore does not need a high level of photosynthesis. In the later days of the incubation, due to the decrease in the carbon source concentration, the amount of chlorophyll a increased photosynthesis efficiency.

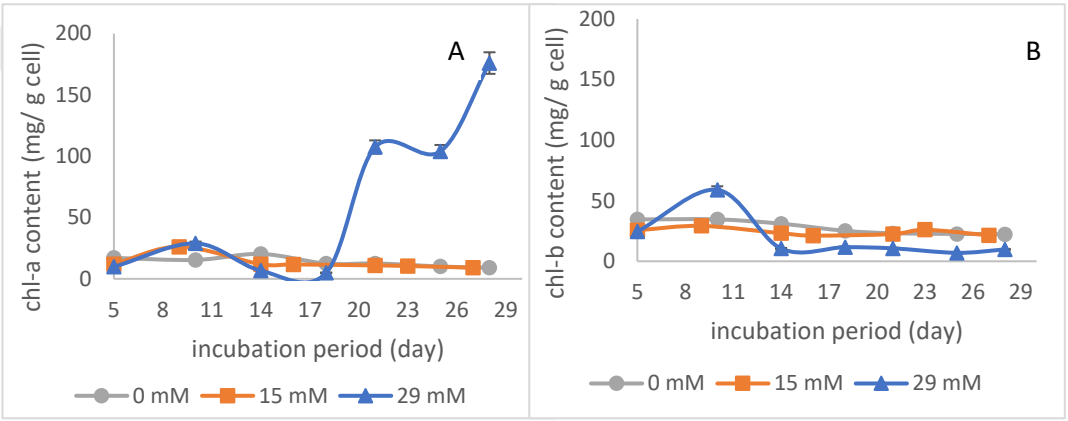

Figure 3.42 Chlorophyll a and chlorophyll b content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different nitrogen concentrations (0, 15 and 29 mM) (A) Chlorophyll a, (B) Chlorophyll b

In high nitrogen containing medium, protein and total carbohydrate values reached high levels in the last days of incubation (Figure 3.43 and 3.44). This may indicate that the cell cannot adapt to media with low nitrogen content. The maximum protein content was determined as 8.86±0.42 mg/ g cell, and the maximum t total carbohydrate content was detected as 0.45±0.02 mg/ g cell ($p<0.05$).

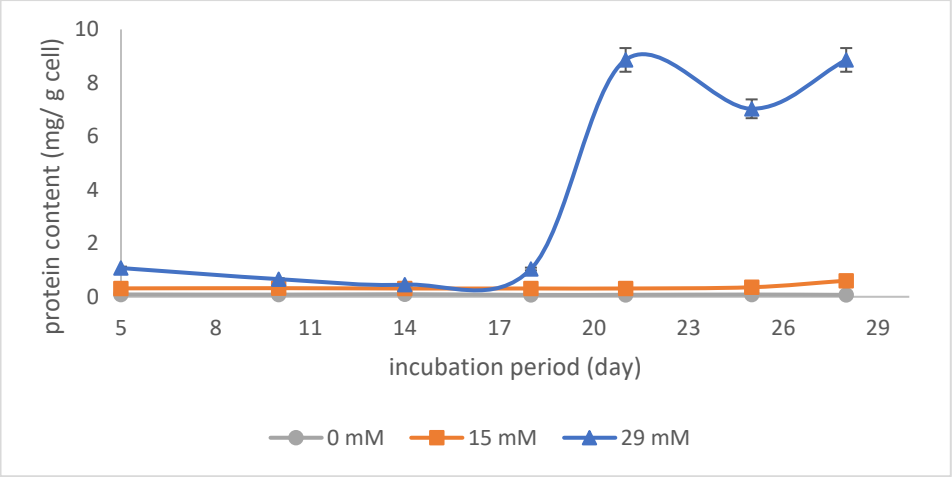

Figure 3.43 Protein content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different nitrogen concentrations (0, 15 and 29 mM)

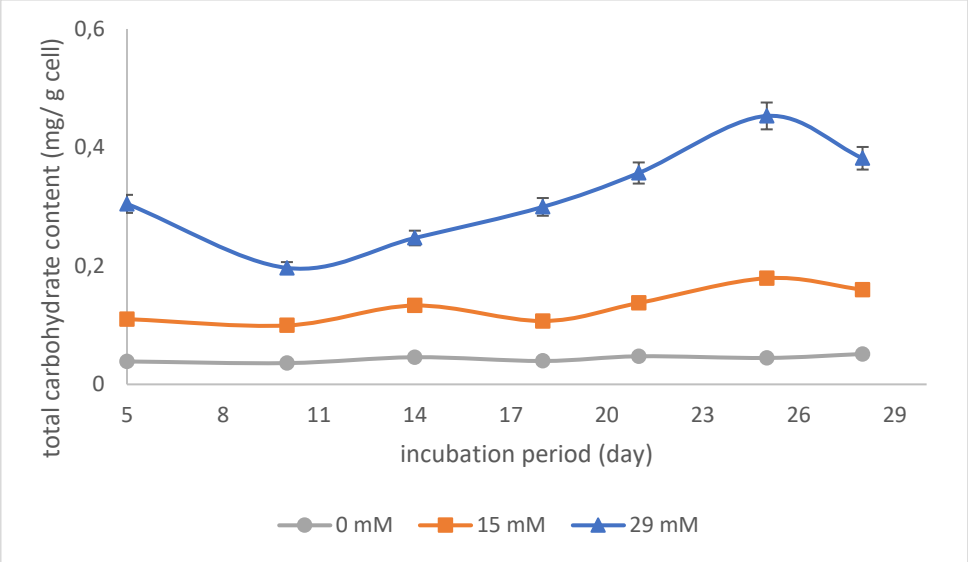

Figure 3.44 Total carbohydrate content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different nitrogen concentrations (0, 15 and 29 mM)

It has been determined that lipid production is high when there is the lowest and the highest nitrogen concentration in the growth medium (Figure 3.45). The maximum lipid production (5.78±0.21 mg/ g cell) was detected with 29 mM nitrogen concentration. This is expected, given that both conditions create stress conditions for microalgae cells. Low nitrogen concentration in the medium triggers lipid production as it will increase the C/N ratio (Goncalves et al., 2016). Similarly, even if the nitrogen concentration in the medium has not changed, the increase in the amount of carbon

will increase the C/N ratio and the lipid production will increase. Because there is not enough nitrogen in the medium for protein synthesis, the fixed carbon is used in the synthesis of storage materials such as TAG. This mechanism comes to the fore in mixotrophic production. The carbohydrate will provide both the pyruvate as the carbon skeleton for lipid synthesis and the ATP and reducing power (NADPH) that will be required in the synthesis (Rawsthorne, 2002). In this respect, although the conditions in which there is no nitrogen in the production environment are thought to be more suitable for lipid production, it has been determined that the production environment containing 29 mM nitrogen is more suitable for the low biomass values in these conditions.

When the data were analyzed by Tukey's HSD test, no significant difference was detected between nitrogen concentration, lipid, protein and carbohydrate content ($p>0.05$). It was determined that there was a significant relationship between lipid, protein and total carbohydrate contents in the environment where the highest lipid production occurred ($p=0.0395$).

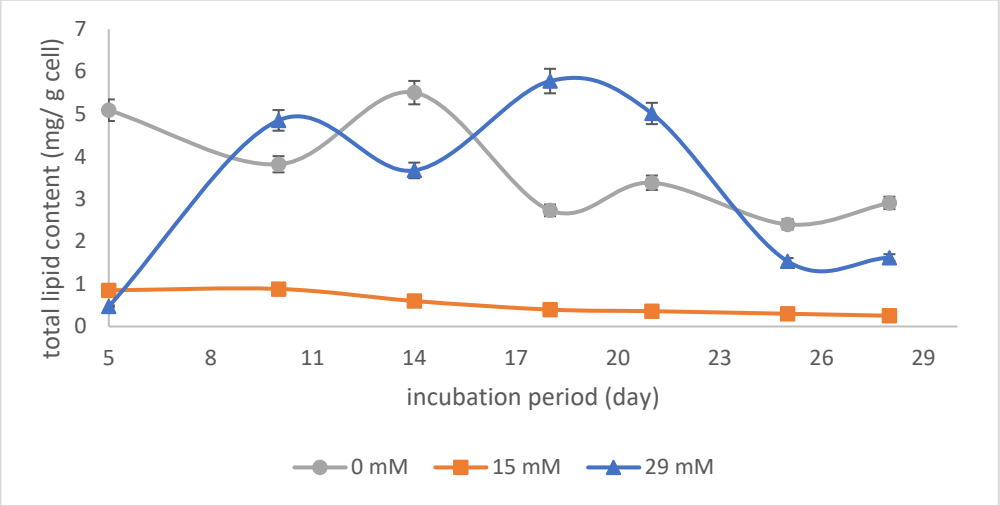

Figure 3.45 Total lipid content changes *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different nitrogen concentrations (0, 15 and 29 mM)

3.3.3 Effects of Light Intensity on H. pluvialis Culture

It was determined that *H. pluvialis* was grown in different trophic conditions and in the presence of different carbon sources in the first stage under mixotrophic conditions and the highest lipid production was detected in the medium containing 2.5 mM crude glycerol. In the next stage, the effect of nitrogen concentration in this medium on the total lipid amount was investigated and the highest lipid production occurred in the medium containing 2.9 mM nitrogen. After determining the carbon source type, concentration and nitrogen concentration in the medium where *H. pluvialis* reached the highest value in terms of total lipid production, the effect of light intensity (1500, 2500 and 4000 lux) on microalgal biomass and total lipid production was examined.

Light is the source of energy in the photoautotrophic growth phase, and microalgae use light energy to convert CO_2 into organic compounds, especially carbohydrates (Juneja et al., 2013). In terms of OD, it was determined that the maximum results in biomass production were reached at 2500 lux light intensity and the specific growth rate of 0.06 day^{-1} in the medium is also the maximum value ($p<0.05$) (Figure 3.46). While microalgal growth rate reaches its maximum value in saturation light intensity, a decrease in growth rate is determined at values of light intensity below or above this value (Juneja et al., 2013). A microalga, which is tried to be grown in a mixotrophic culture, tries to adapt to the environment with photoautotrophic metabolism in the first stage, then consumes the carbon resource available in the growth medium. While it is thought that the adaptation of *H. pluvialis* is difficult in a medium with low light intensity and therefore high OD values cannot be reached, it has been interpreted that the OD cannot reach very high values due to the photoinhibition in the environment with high light intensity. Photoinhibition occurs as a result of breaking down the chloroplast lamella with high light intensity or as a result of the inactivation of enzymes involved in CO_2 fixation (Juneja et al., 2013).

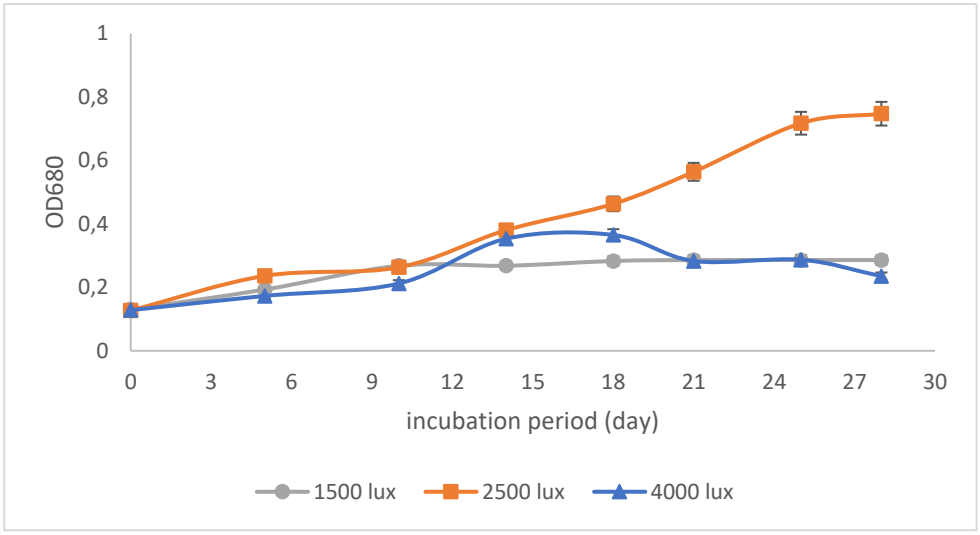

Figure 3.46 OD values of *H. pluvialis* in mixotrophic culture containing 2.5 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

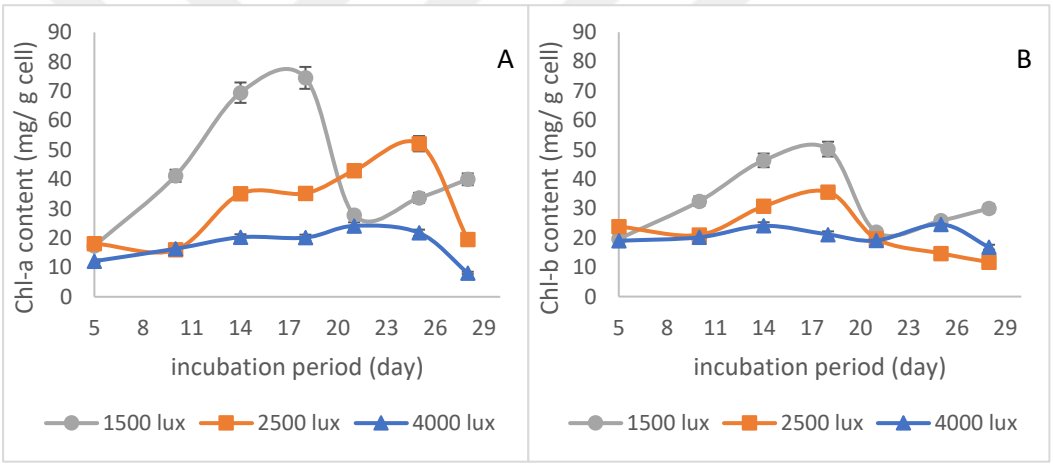

Figure 3.47 Chlorophyll a and chlorophyll b content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux) (A) Chlorophyll a, (B) Chlorophyll b

A certain decrease was detected in the chlorophyll content of *H. pluvialis* grown at varying light intensity, together with increasing light intensity (Figure 3.47). At low light intensity, the microalgae cell increases the amount of chlorophyll in order to capture more photons. With the increase in light intensity, it is not necessary to need a high amount of chlorophyll because it can capture enough photons with less chlorophyll. The maximum chlorophyll a (74.53±0.33 mg/ g cell) and b (50.20±0.22 mg/ g cell) contents were detected with 1500 lux light intensity ($p<0.05$).

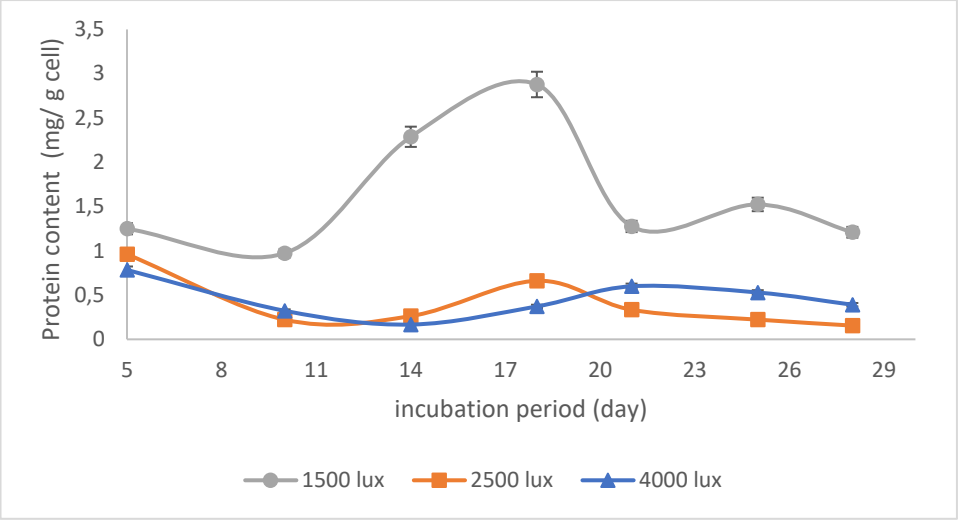

Figure 3.48 Protein content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

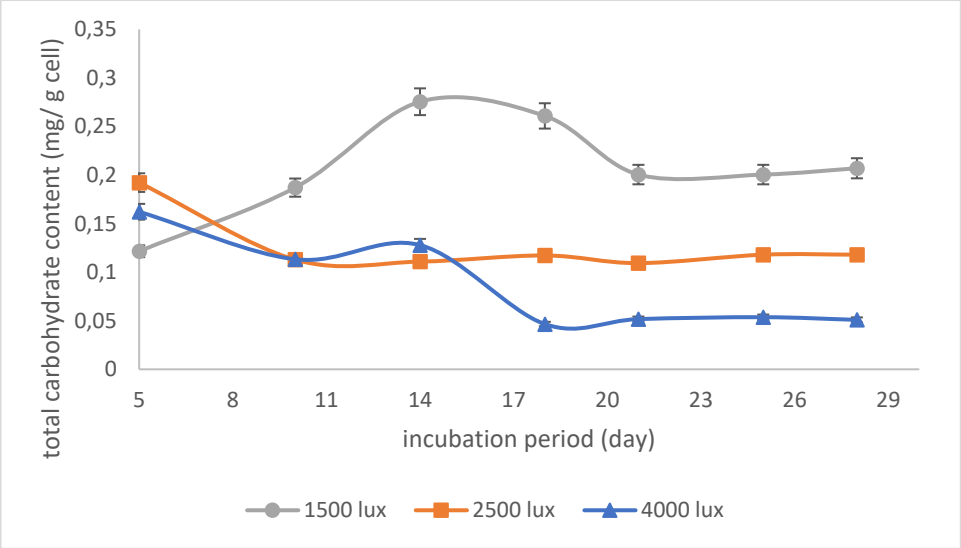

Figure 3.49 Total carbohydrate content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

It has been determined that protein and total carbohydrate levels in these cultures also decrease with increasing light intensity (Figure 3.48 and 3.49). The maximum protein content (2.88±0.13 mg/ g cell) and the maximum carbohydrate content (0.28±0.01 mg/ g cell) were detected with 1500 lux light intensity ($p<0.05$). The reason for the low protein and carbohydrate levels in the medium where high light intensity (4000 lux) is used is thought to be the photoinhibition of the microalgae cell. Due to

the optimum light exposure *for H. pluvialis* growth in medium intensity (2500 lux) light medium, its metabolism may have operated normally and at high levels and did not need to produce and store these metabolites at high levels.

For lipid synthesis, it must first reach the sufficient carbohydrate level in the cell (Ge, Champagne, Plaxton, Leite & Marazzi, 2017). Carbohydrate will provide both pyruvate as the carbon skeleton for lipid synthesis and ATP and reducing power (NADPH) that will be required in the synthesis. In heterotrophic or mixotrophic microalgal cultivation, carbon influx into fatty acids occurs by the production of phosphoenol pyruvate (PEP) by cytosolic glycolysis. After PEP is transported into the plastid, it is converted to pyruvate and then acetyl CoA by pyruvate kinase and pyruvate dehydrogenase enzymes. These metabolites required for fatty acid synthesis are obtained by the breakdown of carbohydrate. For this reason, high carbohydrate level is desired in the targeted microalgae cell for lipid production, the aim is to trigger the flow of carbon in the carbohydrate to lipid production.

The maximum total lipid amount (4.32±0.22 mg/ g cell) was detected on the 14^{th} day of incubation in the presence of 1500 lux light (Figure 3.50). While the total amount of lipid in other media remained lower, its highest values were reached in the late days of incubation. This result shows that both the adaptation of *H. pluvialis* at higher light intensity is later and that photoinhibition may have occurred. This situation is interpreted as a decrease in the amount of cellular lipid as a result of the oxidative stress of polyunsaturated fatty acids by high light intensity. When the data were analyzed by Tukey's HSD test, significant difference was detected between light intensity and lipid, protein production and carbohydrate content ($p=0.0037$). It was determined that there was a significant relationship between lipid, protein and total carbohydrate contents in the environment where the highest lipid production occurred ($p=0.0068$).

It is thought that as the light intensity increases, lipid production will increase due to the formation of photo-oxidative stress. In the study investigating the effect of oxidative stress on lipid production of *Synechocystis* sp cyanobacteria, it was determined that there was no increase in lipid production in cells exposed to oxidative

stress (Sivaramakrishnan & Incharoensakdi, 2018). This situation was interpreted as that the electrons generated by ROS might have been consumed in a different pathway, not in lipid synthesis. For example, in plants, it is known that oxidative stress leads to an increase in soluble sugars. In addition, some studies have shown that low light intensity supports lipid accumulation (Chávez-Fuentes et al., 2018).

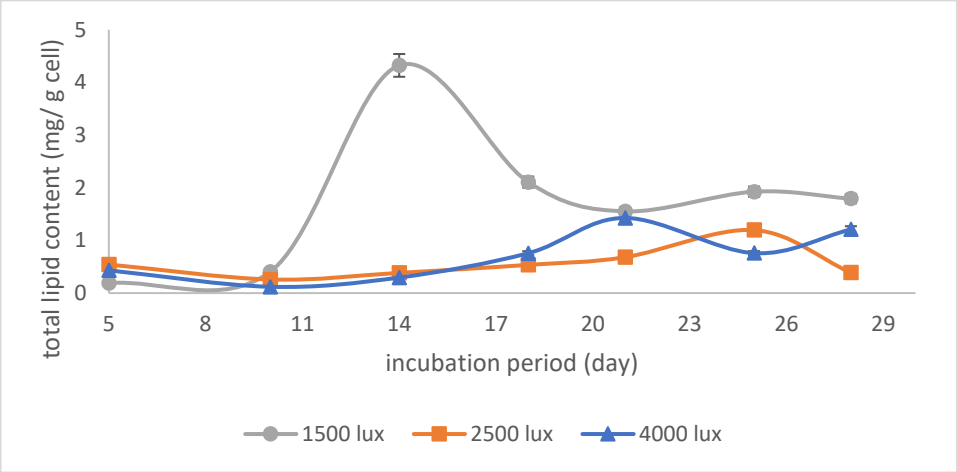

Figure 3.50 Total lipid content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

3.3.4 Effects of Light Intensity on S. platensis Culture

In the first stage of the study, suitable carbon source and cultivation type for lipid production of *S. platensis* were determined as mixotrophic culture containing 10 mM crude glycerol. Then, the effect of nitrogen amount on lipid production was investigated and the highest lipid production was determined in the presence of 29 mM nitrogen. After determining the carbon source type, concentration and nitrogen concentration in the medium where *S. platensis* reached the highest value in terms of total lipid production, the effect of light intensity (1500, 2500 and 4000 lux) on microalgal biomass and total lipid production was examined.

The maximum OD and specific growth rate (μ= 0.31 day^{-1}) were reached in the presence of 1500 lux light intensity ($p<0.05$) (Figure 3.51). In a similar study, it was concluded that *S. platensis* was able to assimilate the glycerol in the growth medium more effectively at low light intensity (Markou et al., 2019). For this reason, the best

growth was obtained at the lowest light intensity. At higher light intensity, cell growth remained low due to a possible photoinhibition. A similar situation is observed in chlorophyll amounts (Figure 3.52). In the lowest light intensity environment, the maximum chlorophyll a (175.98±0.86 mg/ g cell) and b (58.96±0.26 mg/ g cell) values were obtained ($p<0.05$). This result may have been encountered as a lower amount of light capture apparatus at high light intensity is needed. It may also be due to the deterioration of PS II as a result of photoinhibition.

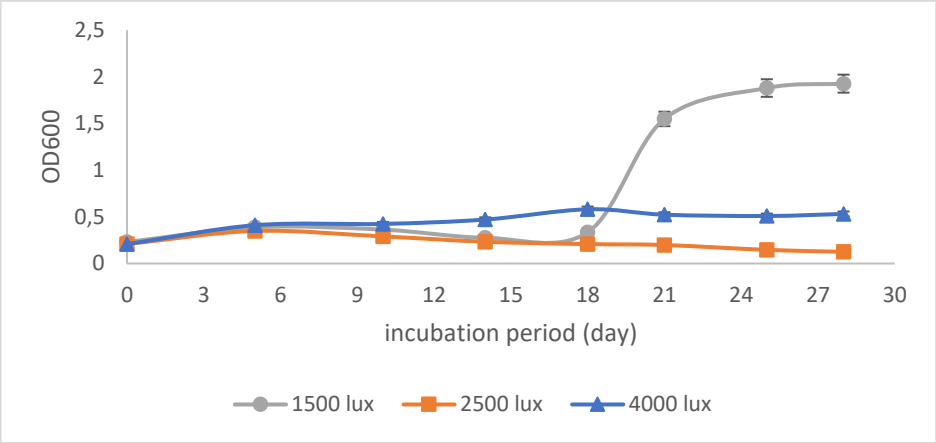

Figure 3.51 OD values of *S. platensis* in mixotrophic culture containing 10 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

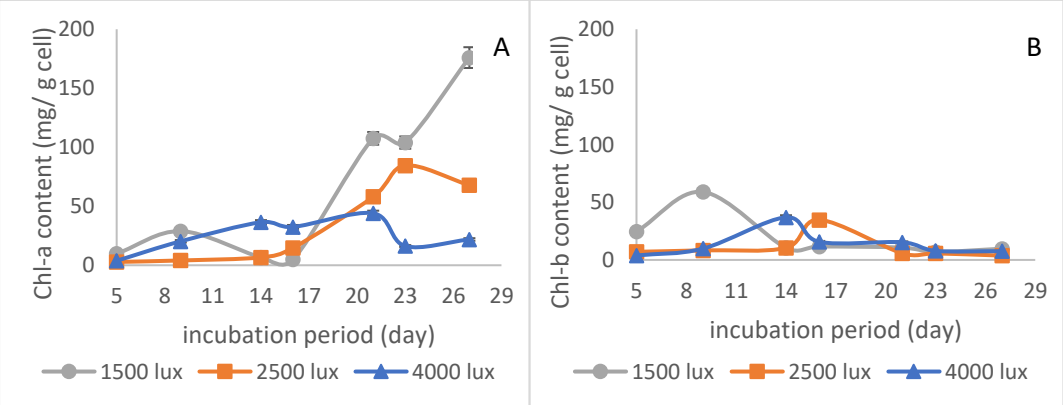

Figure 3.52 Chlorophyll a and chlorophyll b content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux) (A) Chlorophyll a, (B) Chlorophyll b

The maximum protein (8.86±0.42 mg/ g cell) (Figure 3.53) and total carbohydrate (0.45±0.02 mg/ g cell) (Figure 3.54) values were also determined at 1500 lux light intensity. This result is also supported by the OD values. Since carbohydrates are the basic products of photosynthesis, the amount of carbohydrates is expected to be high in the environment where photosynthetic activity is high (Markou et al., 2019). This situation can be interpreted as the *S. platensis* was insufficient to adapt to high light intensity and the photoinhibition effect occurs. The maximum lipid level (5.78±0.21 mg/ g cell) was detected with 1500 lux light intensity on 18th day of incubation ($p<0.05$) (Figure 3.55). A similar result was obtained in another study, it was determined that the amount of lipid decreased with increasing light intensity (Markou et al., 2019).

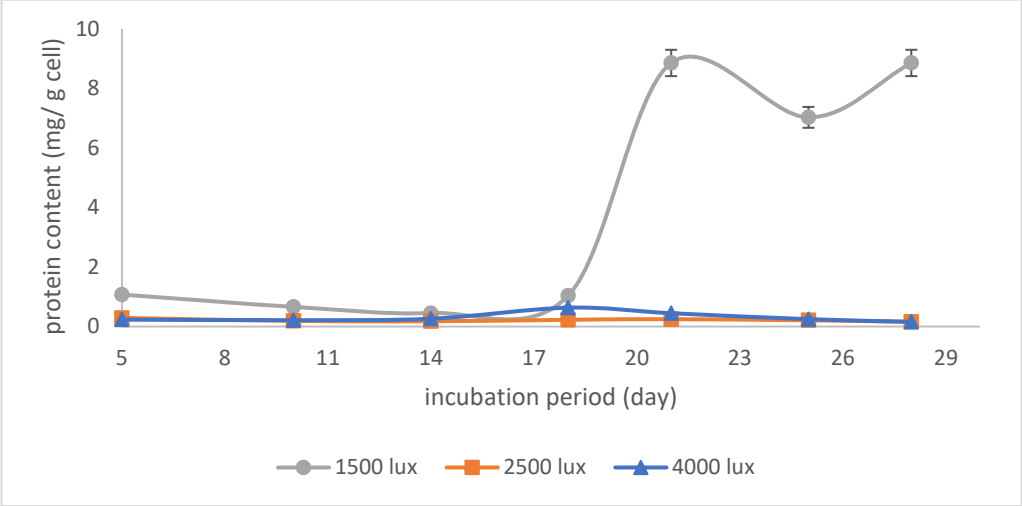

Figure 3.53 Protein content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

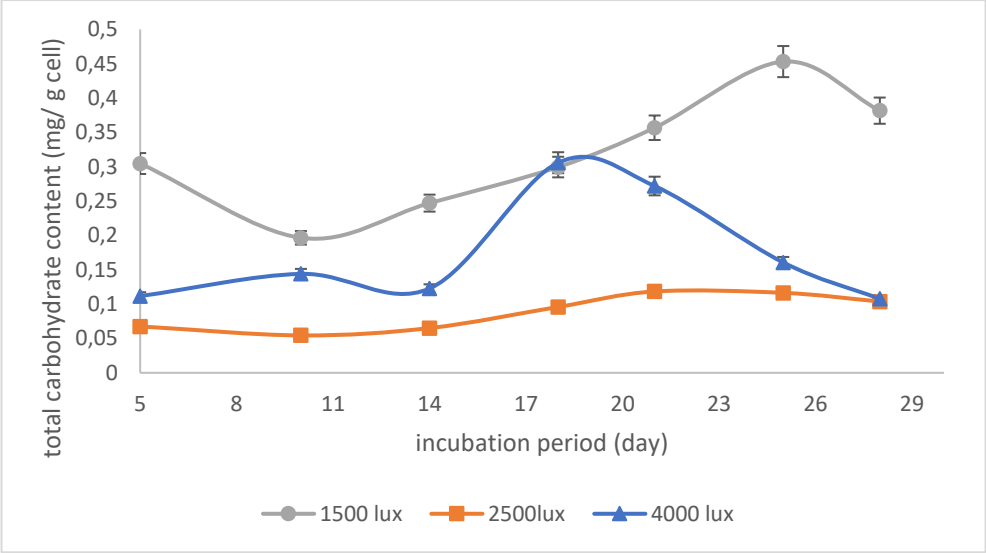

Figure 3.54 Total carbohydrate content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

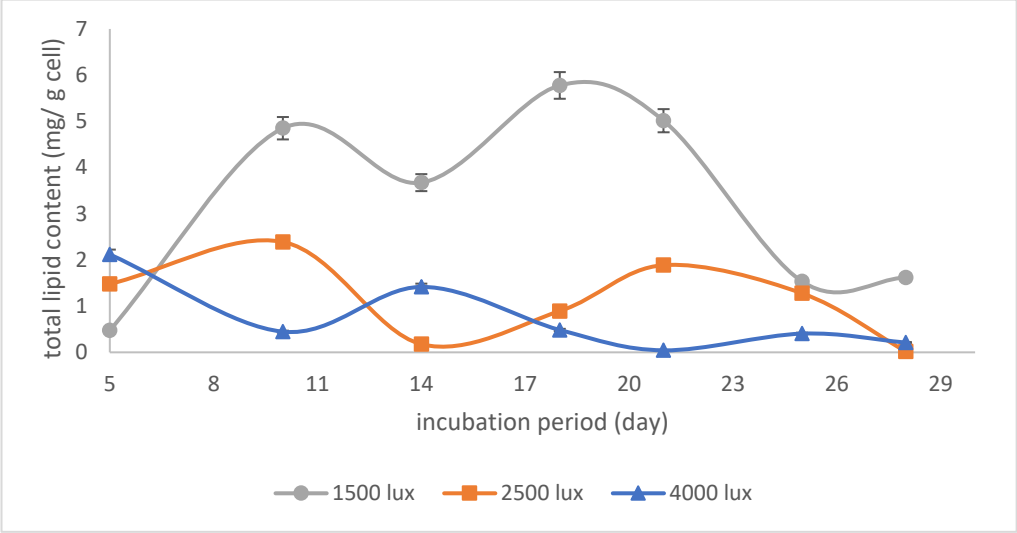

Figure 3.55 Total lipid content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different light intensities (1500, 2500 and 4000 lux)

The results obtained can be interpreted as light intensities above 1500 lux for *S. platensis* cause photoinhibition and affect all metabolic pathways in the cell. Additionally, the analyzed data by Tukey's HSD test, shows significant difference between light intensity and lipid, protein production and carbohydrate content ($p=0.0037$).

3.3.5 Effects of Phosphorus Concentration on H. pluvialis Culture

After determining the optimum conditions of carbon source type and concentration, nitrogen concentration and light intensity parameters for lipid production of *H. pluvialis*, the effect of phosphorus concentrations (0, 2.5 and 5.6 mM) was investigated. It is a vital nutrient in the production of microalgae such as nitrogen and carbon, which is involved in the structure of various energy carrier molecules, phospholipids and nucleic acids. It takes part in many important bioprocesses such as energy transfer, signal transmission, photosynthesis and respiration (Liang et al., 2013).

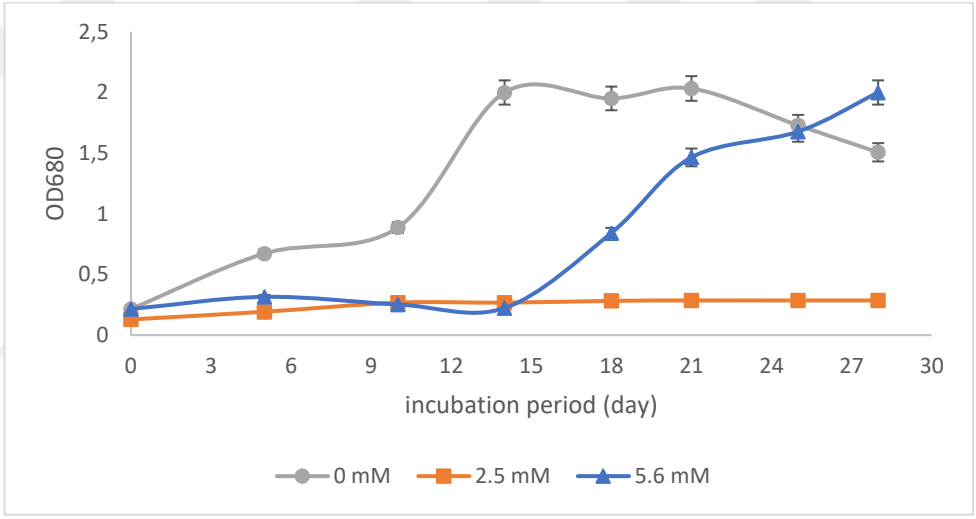

Figure 3.56 OD values of *H. pluvialis* in mixotrophic culture containing 2.5 mM crude glycerol with different phosphorus concentration (0, 2.5 and 5.6 mM)

The maximum specific growth rate (μ= 0.08 day^{-1}) and OD were obtained under conditions where 5.6 mM phosphorus was present in the growth medium ($p<0.05$) (Figure 3.56). When the phosphorus concentration in the growth medium decreases, cell division stops; however, cell growth continues (Wu et al., 2015). For this reason, OD was determined to be low in environments containing lower phosphorus concentrations.

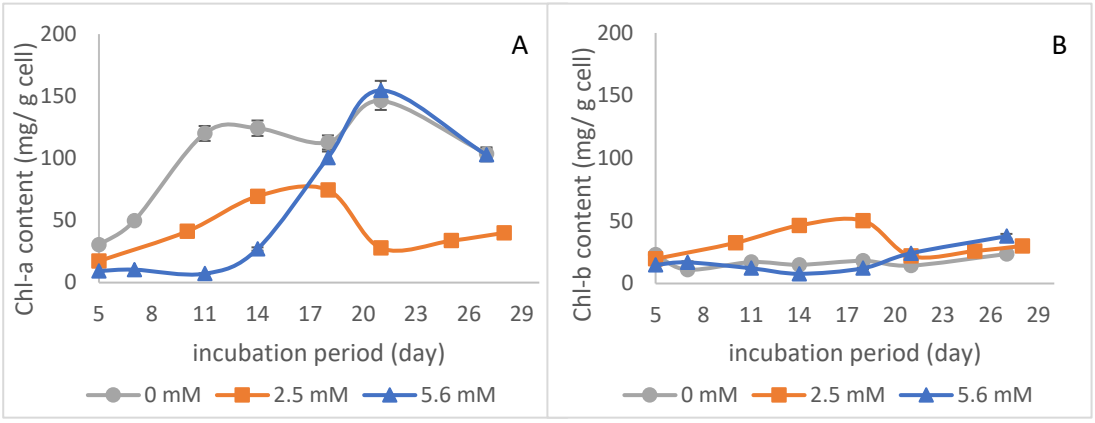

Figure 3.57 Chlorophyll a and chlorophyll b content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different phosphorus concentration (0, 2.5 and 5.6 mM) (A) Chlorophyll a, (B) Chlorophyll b

It was determined that the level of chlorophyll a in *H. pluvialis* grown in different phosphorus concentrations in the absence of phosphorus and in excess phosphorus reached high values (Figure 3.57). The maximum chlorophyll a (154.66±7.36 mg/ g cell) content was detedted in the presence of 5.6 mM phoshorus while the maximum chlrophyll b (50.20±0.22 mg/ g cell) content was determined with 2.5 mM phosphorus concentration ($p<0.05$). However, while the amount of chlorophyll a remained at low values in the medium where phosphorus was 2.5 mM, the amount of chlorophyll b reached the maximum levels. Phosphorus deficiency affects chlorophyll synthesis, reducing the level of photosynthetic phosphorylation, ATP synthesis and Calvin cycle efficiency (Liang et al., 2013). The reduction in photosynthesis in media without nitrogen and phosphorus is due to the degradation of light-dependent proteins such as the cytochrome b6f complex, and ATP synthase and PS II proteins help cell survival by reducing ROS (Arora, Patel, Pruthi & Pruthi, 2016). When the amount of nitrogen and phosphorus increases, the sum of chlorophyll a and chlorophyll b increases.

The maximum protein content (2.88±0.13 mg/ g cell) was determined with 2.5 mM phosphorus concentration ($p<0.05$) (Figure 3.58). The absence of phosphorus in the growing environment will adversely affect protein production. The presence of high amounts of phosphorus in the production medium may have inhibited protein production. The total carbohydrate content did not change significantly during the

incubation period in all three media ($p>0.05$) (Figure 3.59). The maximum total carbohydrate content was detected as 0.43±0.02 mg/ g cell ($p<0.05$).

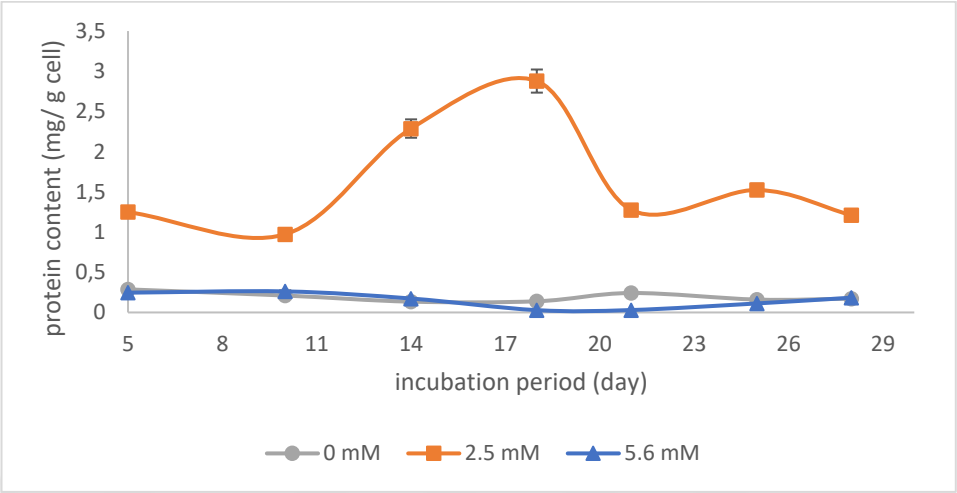

Figure 3.58 Protein content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different phosphorus concentration (0, 2.5 and 5.6 mM)

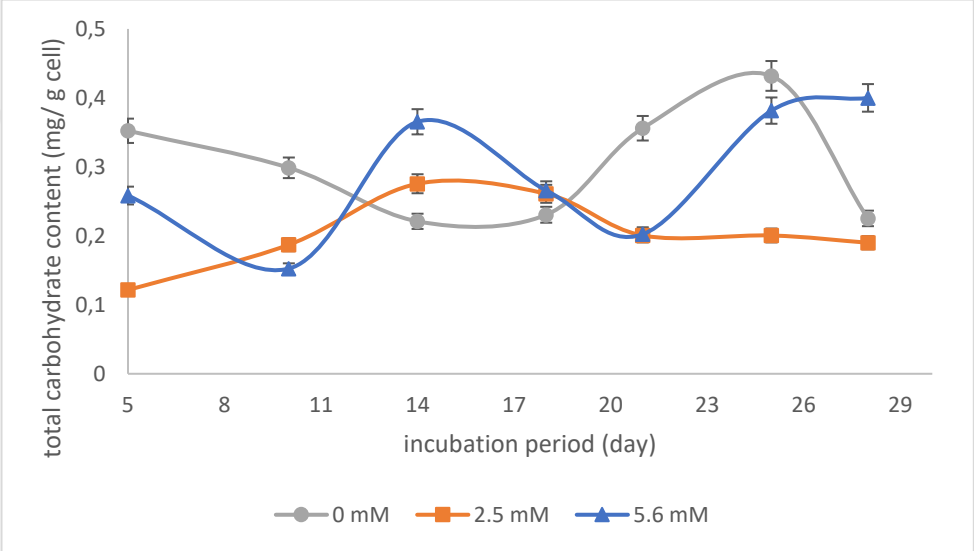

Figure 3.59 Total carbohydrate content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different phosphorus concentration (0, 2.5 and 5.6 mM)

When the lipid levels produced due to the changing phosphorus concentration were examined, it was determined that the lipid production decreased in parallel with the decrease in the phosphorus concentration (Figure 3.60). The highest lipid amount (11.49±0.57 mg/ g cell) was detected on the 5th day of incubation in production culture

including 5.6 mM phosphorus ($p<0.05$). This value is approximately 2.66-fold higher than non-optimized conditions and 12 times greater than the value of lipid produced in photoautotrophic cultivation ($p<0.05$). Additionally, in this optimized condition the lipid production process became faster about 9 days. The optimum lipid production condition was detected as mixotrophic culture which contains 2.5 mM crude glycerol, 2.9 mM nitrogen, 5.6 mM phosphorus and illuminated with 1500 lux light intensity (Table 3.3). In this conditions C/N/P ratio was 9:4.1:41 and lipid productivity was 9.19 mg/L.d. It can be said that the stress caused by high phosphorus concentration triggers lipid production. In general, the cell needs energy in the form of ATP for biomolecule synthesis. The high level of phosphorus may have had a positive effect on the synthesis of fatty acids.

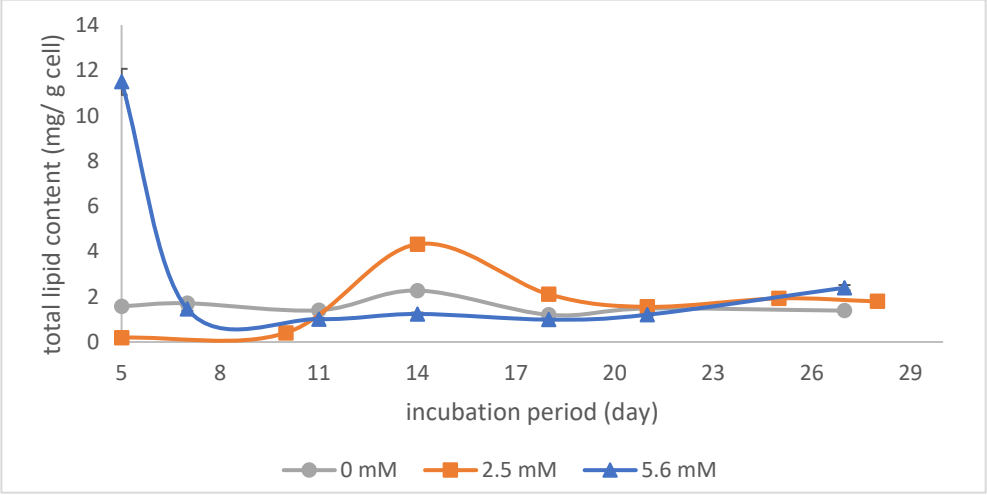

Figure 3.60 Total lipid content changes of *H. pluvialis* grown in mixotrophic culture containing 2.5 mM crude glycerol with different phosphorus concentration (0, 2.5 and 5.6 mM)

Interaction between phosphorus concentration and lipid production is species specific (Yu et al., 2019). The results obtained show that high phosphorus concentration triggers lipid production. Excess electrons accumulating from the photosynthetic electron transport chain under environmental stress can trigger the overproduction of ROS. The presence of phosphate in the growth medium creates a synergetic effect on nitrate supplying nitrate entering the cell (Saadaoui et al., 2018). This indicates that the cell is not challenged in terms of nitrogen uptake in the presence of high phosphorus. Thus, it can direct its energy and existing carbon skeleton to lipid production. The production of C18 fatty acids causes about 24 NADPH to be used

from the electron transport chain, thus relaxing the electron transport chain under stress (Wu et al., 2015). For this reason, the cell may have protected itself with lipid production from the stress condition created by high phosphorus concentration. In the following days of the incubation, it is expected that the produced lipid will be converted into other components such as protein and carbohydrate.

Table 3.3 Optimum conditions of lipid production medium for *H. pluvialis*

Carbon source type and concentration	Crude glycerol, 2.5 mM
Nitrogen concentration	2.9 mM (BBM concent.)
Phosphorus concentration	5.9 mM (higher than BBM concent.)
C:N:P	9: 4.1: 41
Light intensity	1500 lux
Produced lipid level	11.49±0.57 mg/ g cell
Lipid Productivity	9.19 mg/L.d
Specific growth rate	0.08 day^{-1}
Compare with photoautotrophic culture	12.22 fold higher ($p<0.05$)

LPO and proline levels were investigated by extracting the lipid produced under optimum conditions. Under various nutritional or environmental stresses, microalgae cells change their LPO, which indicates oxidative degredation of lipids, and proline, which is an element of stabilization mechanism, levels to protect themselves against stress condition. LPO values of control (0.29±0.01 μM MDA/g cell) and optimized (0.24±0.01 μM MDA/g cell) medium conditions support this result. In the present study, a similar LPO value was found in optimized condition, which suggests the protective role of produced lipids. The produced lipid contains neutral lipids such as TAG and this form of lipids increases membrane stability of microalgae cell (Lu, Wei, Jiang, Chen & Yang, 2012). Consistent with our study, Bilbao, Damiani, Salvador & Leonardi (2016) found that LPO did not change in *H. pluvialis* after 3 days of light-induced stress and that TAG content also increased. Also, in this stress state, the proline value of produced cell in optimized medium was detected as 10.10±0.47 μmol/ g cell and in the control condition it was detected as 0.94±0.04 μmol/g cell. Proline helps in stabilization of subcellular structures and it is known to accumulate under

various environmental and nutritional stress conditions (Esen & Ozturk Urek, 2015; Ozturk Urek & Kerimoglu, 2019).

Later in the incubation in optimized medium, it is expected that the produced lipid will be converted to other components such as protein and carbohydrate. When the amount of phosphorus in the growth medium decreases, cell division stops; however, cell growth continues (Wu et al., 2015). This may be the reason causing low protein values detected at high phosphorus concentration. In the phosphorus-free medium, low protein production was realized due to the negative effect of energy metabolism. The highest lipid content was reached with phosphorus stress, and the carbohydrate content was also at a certain level under these conditions. The excess carbohydrate produced under these conditions acts as structural components of the cell wall (Markou, Angelidaki & Georgakakis, 2012). This situation can be interpreted as it may help decrease LPO in the environment where the highest lipid production is determined. The LPO levels given above also support this interpretation. Additionally, consistent with the results obtained in this study, it was reported that phosphorus starvation and carbohydrate production increased while protein production decreased (Markou et al., 2012). When the data were analyzed by Tukey's HSD test, no significant difference was noted between phosphorus concentration, lipid production, lipid and carbohydrate content ($p>0.05$). There was a moderate positive correlation between protein and lipid production ($r=0.5513$).

3.3.6 Effects of Phosphorus Concentration on S. platensis Culture

After determining the optimum conditions of carbon source type and concentration, nitrogen concentration and light intensity parameters for lipid production of *S. platensis*, the effect of phosphorus concentration (0, 1.5 and 2.9 mM) was investigated. The maximum OD value and specific growth rate ($\mu= 0.31$ days^{-1}) were determined in culture including 1.5 mM phosphorus ($p<0.05$) (Figure 3.61). In the absence of phosphorus in the growth medium, cell division is inhibited, while high phosphorus concentration can also inhibit growth due to the stress it creates. For example, in a study investigating the lipid production potential of *C. vulgaris* by growing it in

different media, it was determined that 0.2 g/ L phosphorus concentration inhibits growth (Li et al., 2016).

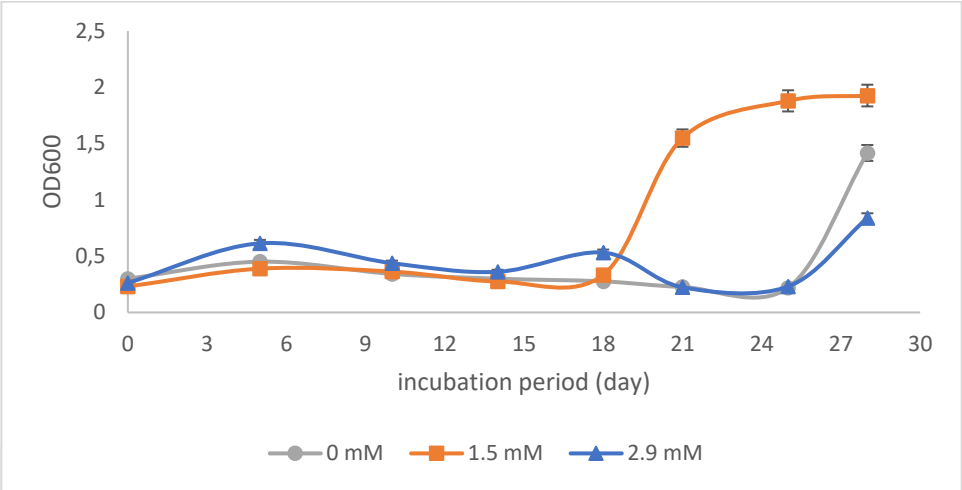

Figure 3.61 OD values of *S. platensis* in mixotrophic culture containing 10 mM crude glycerol with different phosphorus concentration (0, 1.5 and 2.9 mM)

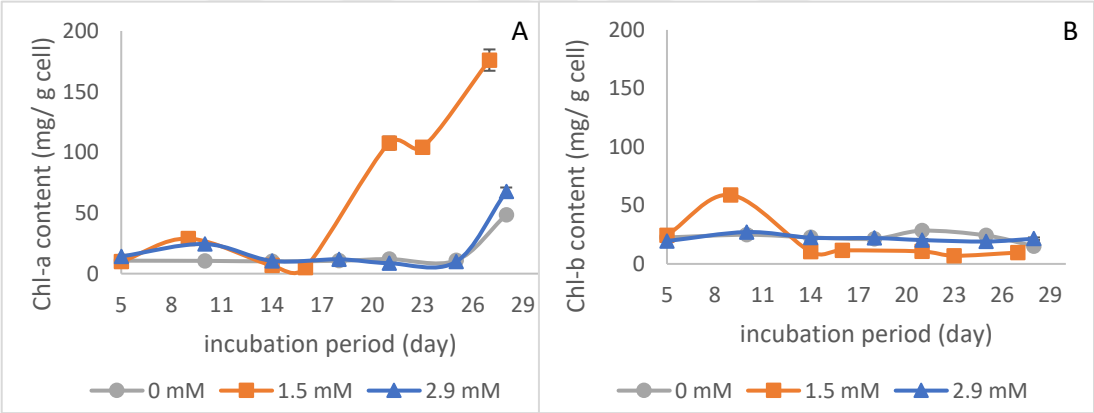

Figure 3.62 Chlorophyll a and chlorophyll b content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different phosphorus concentration (0, 1.5 and 2.9 mM) (A) Chlorophyll a, (B) Chlorophyll b

Chlorophyll values were also low in media containing 2.9 mM phosphorus and no phosphorus in which biomass production is low (Figure 3.62). However, in the medium containing 1.5 mM phosphorus, it was determined that the amount of chlorophyll b was high in the first days of incubation and the amount of chlorophyll a after the 17th day. This situation shows that *S. platensis* in mixotrophic culture first uses its external carbon source and increases photosynthesis activity after its exchange

decreases. The maximum chlorophyll a (175.98±0.86 mg/ g cell) and b (58.96±0.26 mg/ g cell) contents were detected with 1.5 mM phosphorus content ($p<0.05$).

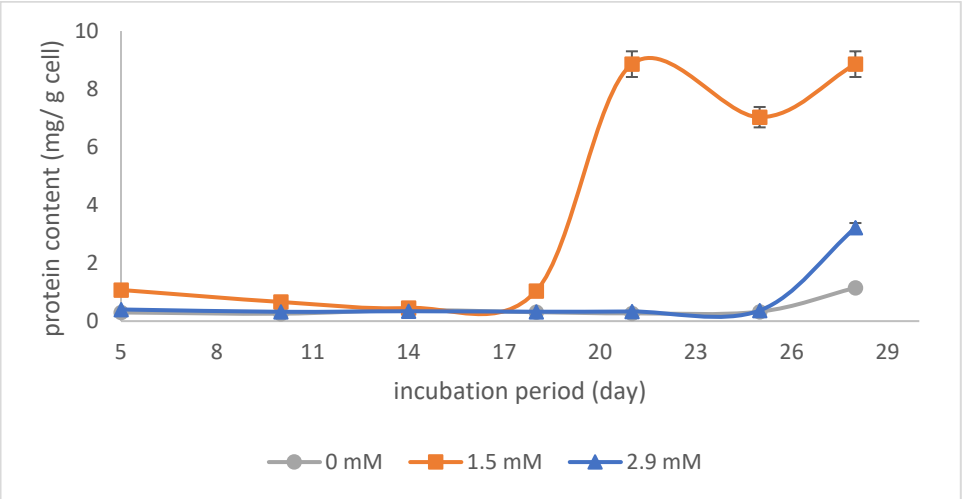

Figure 3.63 Protein content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different phosphorus concentration (0, 1.5 and 2.9 mM)

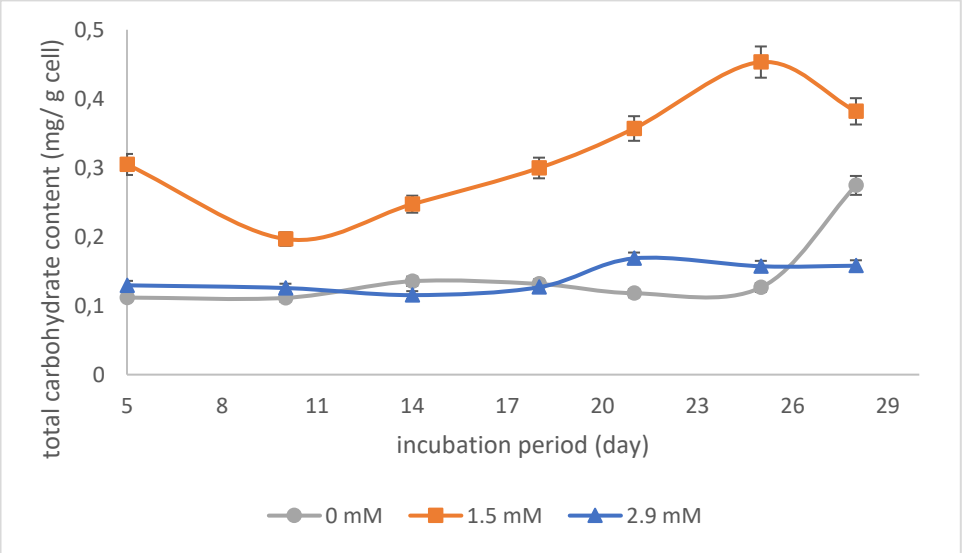

Figure 3.64 Total carbohydrate content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different phosphorus concentration (0- 1.5 and 2.9 mM)

The maximum protein (8.86±0.42 mg/ g cell) (Figure 3.63) and total carbohydrate (0.45±0.02 mg/ g cell) (Figure 3.64) contents were detected in the presence of 1.5 mM phosphorus ($p<0.05$). This situation can be interpreted as the microalgae cannot adapt to the conditions of phosphorus deficiency and phosphorus excess.

When the change in lipid production due to phosphorus concentration was examined, it was determined that the highest lipid amount (5.78±0.21 mg/ g cell) was obtained in the medium containing 1.5 mM phosphorus ($p<0.05$) (Figure 3.65). When the data were analyzed by Tukey's HSD test, no significant difference was detected between phosphorus concentration, lipid, protein and carbohydrate content ($p>0.05$).

The highest lipid production was deteemined in mixotrophic medium that contains 10 mM crude glycerol and the produced lipid level was about 19.17% higher than the photoautotrophic (control) condition ($p<0.05$). The optimum lipid production condition was detected as mixotrophic culture which contains 10 mM crude glycerol, 29 mM nitrogen, 1.5 mM phosphorus and illuminated with 1500 lux light intensity (Table 3.4). In this condition C/N/P ratio was 36:4.1:1.13 and lipid productivity was 1.28 mg/L.d. It can be concluded that the stress caused by high carbon and low nitrogen concentration triggers lipid production. Lipid production remained lower in conditions where there was no phosphorus or 2.9 mM phosphorus in the production environment. Similarly, in a study examining lipid production with *Botrycoccus braunii*, the highest lipid value was determined in medium containing 222 mg/ L phosphorus, while lipid values remained low in media containing lower and higher phosphorus values (Ruangsomboon, 2012).

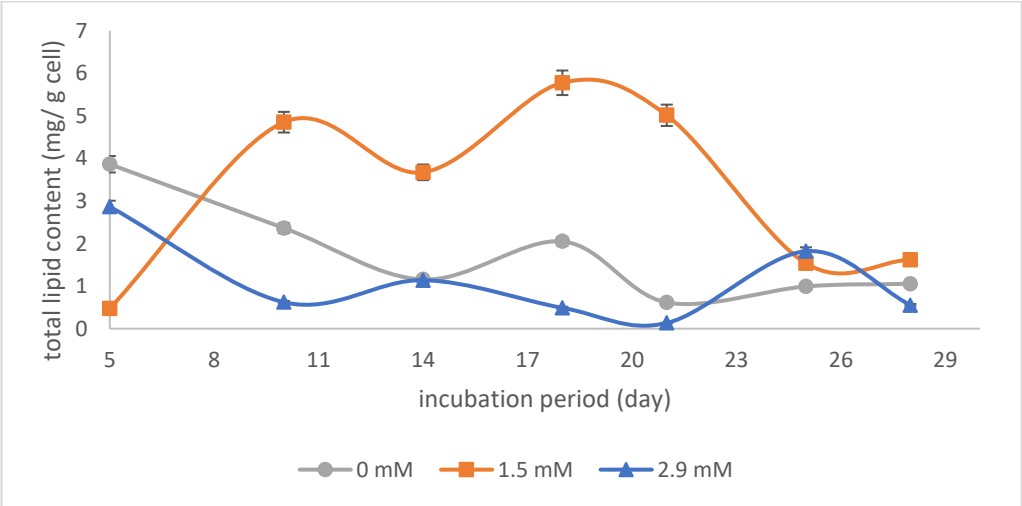

Figure 3.65 Total lipid content changes of *S. platensis* grown in mixotrophic culture containing 10 mM crude glycerol with different phosphorus concentration (0, 1.5 and 2.9 mM)

Table 3.4 Optimum conditions of lipid production medium for *S. platensis*

Carbon source type and concentration	Crude glycerol, 10 mM
Nitrogen concentration	29 mM (Zarrouk's medium concent.)
Phosphorus concentration	1.5 mM (Zarrouk's medium concent.)
C:N:P	36: 4.1: 1.13
Light intensity	1500 lux
Produced lipid level	5.78±0.21 mg/ g cell
Lipid Productivity	1.28 mg/L.d
Specific growth rate	0.31 day^{-1}
Compare with photoautotrophic culture	1.19 fold higher ($p<0.05$)

Phosphorus deficiency disrupts the normal function of the phytoplankton cell, so new metabolic pathways are formed. Lipid synthesis is catalyzed by ACCase, this enzyme converts acetyl-CoA to malonyl-CoA and fatty acids are formed upon completion of the cycle. Meanwhile, lipid biosynthesis has been attributed to fatty acids and sn-glycerol-3-phosphate. ACCase generally catalyzes the initial reaction of the fatty acid biosynthesis pathway. If there is a lack of phosphorus in the environment, cell division slows down. Excess carbon source is absorbed by the cell and enters the Krebs cycle, which will trigger TAG biosynthesis (Liang et al., 2013). Similarly, it was found that lipid production was higher in medium with less phosphorus.

3.4 FAME Content and Biodiesel Potential of Produced Lipid from *H. pluvialis*

The effect of various environmental and nutritional variables was examined and the lipid produced under the optimum condition was extracted by two different methods and the FAME composition was determined by GC. The FAME composition produced from *H. pluvialis* by different methods is given in Table 3.5.

Table 3.5 FAME composition of produced lipid from *H. pluvialis* with different methods

Type of Fatty Acid	**Percentage of Fatty Acid (Method-1)**	**Percentage of Fatty Acid (Method-2)**
C20:0	n.d.	10.10±0.36
C18:0	32.07±1.72	28.30±1.28
C16:0	11.60±0.43	16.50±0.77
C18:2n6t	8.30±0.27	8.90±0.31
C22:0	6.75±0.22	n.d.
C18:1n9t	6.48±0.21	19.90±1.11
C18:3	6.31±0.21	n.d.
C18:2n6c	6.03±0.18	n.d.
C14:0	4.89±0.13	10.30±0.51

According to Method 1, SUFA content is 55.67±2.50%, PUFA content is 20.64±0.66% and MUFA acid content is 6.48±0.21%. According to Method 2, SUFA content is 65.20±2.92%, PUFA content is 8.90±0.31% and MUFA content is 19.90±1.11%. The high degree of unsaturation of fatty acids provides a high oxidation tendency, which is a desirable property for fuels. However, short and polyunsaturated fatty acids cause increased viscosity, and therefore flow characteristics change at low temperatures (El-Sheekh, Abomohra, Eladel, Battah & Mohammed, 2018). Therefore, the degree of unsaturation and the number of carbons are important criteria. According to the FAME composition detected by Method 1, the degree of unsaturation was 47.76±2.13 and the long chain saturation factor was calculated as 27.36±1.32. According to the FAME composition detected by Method 2, the degree of unsaturation was 37.70±1.81 and the long chain saturation factor was calculated as 25.80±1.26.

Absence of parinaric acid (C18: 4) in fatty acids is important for providing oxidative stability of biodiesel (El-Sheekh et al., 2018). Palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids with carbon atoms of 16-18 are essential fatty acids in biodiesel production (Knothe, 2008).

In a study, *Chlorella* sp. and *S. platensis* microalgae produced fatty acid profile were examined (Dehaghani & Pirouzfar, 2018). It was detected that *S. platensis* produced 59.3% of C16:0, 4.5% of C18:0 and no C14:0. *Chlorella* sp. was found to have fatty acid content 27.8% of C14:0, 11.5% of C16:0 and 1.5% of C18:0. In a study examining the effect of changes in production conditions on fatty acid composition, *Botryococcus braunii* produced approximately 35% of C18:1, 25% of C16:0, 15% of C18:3 and 10% of C18:0 under photoautotrophic conditions (Sadeghin et al., 2018). Compared to these results, it is suggested that the fatty acid profile of the lipid produced in the present study is suitable for fuel production.

Some parameters related to fuel properties of lipid produced under optimized conditions were investigated (Table 3.6). IV, showing the oxidative stability of the fuel, and CN, which considered as an indicator of ignition quality of fuels, have been determined in accordance with European Standards (UNE-EN 14214) and American Standards (ASTM 675).

Table 3.6 Biodiesel potential parameters of produced lipid from *H. pluvialis*

	According to Method-1	**According to Method-2**	**European Standard (UNE-EN 14214)**	**American Society for Testing and Materials (ASTM 675)**
IV (g I_2/100 g)	49.05±2.43	34.01±1.66	<120	-
CN	68.05±3.07	262.4±12.48	>51	>47
SV	166.48±8.11	192.31±8.76	-	-
DU	47.76±2.13	37.70±1.81	-	-
LCSF	27.36±1.32	25.80±1.26	-	-
OS	9.60±0.42	6.32±0.27	>6	>3

This result shows that the saturated fatty acid content is high, which means that the fuel will have an effective combustion property (Chávez-Fuentes et al., 2018). The saturation ratio of a lipid and the length of the chain are highly influential variables in the combustion quality of biofuel. The cetane number and IV of biodiesel produced

from *Leptolyngbya* sp. cyanobacteria was determined as 52.13 and 84.08, respectively and these values were found to be suitable for quality fuel (Tiwari, Bhunia, Bandyopadhyay & Oinam, 2020). The produced biodiesel from *Rhodotorula glutinis* grown in the presence of glucose has an IV of 94.18 and a CN of 51.75 (Viñarta, Angelicola, Van Nieuwenhove, Aybar & de Figueroa, 2020). In another study, *C. vulgaris* microalgae were grown in different light colors and light intensities and CN was determined as 48 and IV was varied between 20 and 100 (Chávez-Fuentes et al., 2018). Similarly, the lipid produced in the thesis study has suitable properties for high quality fuel production.

3.5 FAME Content and Biodiesel Potential of Produced Lipid from *S. platensis*

The FAME content was determined by extracting lipid from the optimized medium (Table 3.7). According to Method 1, SUFA level is 38.31±0.20%, PUFA level is 42.69±2.15% and MUFA level is 18.96±0.91%. According to Method 2, SUFA content is 47.40±2.20%, PUFA content is 9.91±0.47% and MUFA content is 25.60±1.21%. The high PUFA level approves the produced lipid to be utilized as a starvation additive besides its biodiesel characteristic. Even though *S. platensis* is known as a protein producer, these cyanobacteria have important nutritional biomolecules such as polyunsaturated fatty acids.

The generated lipid from *S. platensis* had the main fatty acids such as palmitic, stearic, oleic and linoleic acids which are necessary for quality biodiesel generation. Morais et al. (2019) cultivated *Spirulina* sp. with various levels of glycerol and detected that *Spirulina* sp. has 16-18 carbons fatty acid as basic fatty acid content (89.93%). Likewise, in this thesis study a meaningful section of the total fatty acid composition is 16-18 carbons ($p<0.05$).

Table 3.7 FAME composition of produced lipid from *S. platensis* with different methods

Type of Fatty Acid	**Fatty Acid Content (%) (Method-1)**	**Fatty Acid Content (%) (Method-2)**
C22:0	13.96±0.41	n.d.
C18:3	21.69±1.02	n.d.
C18:2n6c	21.03±1.13	9.91±0.47
C18:1n9t	18.96±0.91	25.60±1.21
C18:0	20.74±1.00	17.77±0.88
C16:0	3.61±0.02	29.63±1.32

Some fuel properties of the produced *S. platensis* lipid were examined according to detected FAME composition (Table 3.8). DU was 104.34±4.93 and the LCSF was detected as 31.67±1.06 in method-1. These values were determined as 45.42±1.77 and 11.85±0.46 in method-2. The lipids with high SV, an indicator of purity of lipid, need higher methanol volumes and produce higher by product (Bart, Palmeri & Cavallaro, 2010). In thesis study the SV was calculated as 194.81±9.03 and 170.22±8.07 in method-1 and method-2, respectively. SV value of produced lipid from *Synechocystis* sp., which was cultivated with glycerol supplementation, was detected as 211.29 (Sivaramakrishnan & Incharoensakdi, 2018). IV, an index of oxidative stability, was likewise European Standards (UNE-EN 14214) that was produced from *S. platensis*. El-Sheekh et al. (2018) reported IV value as 110.37 g I_2/100 g that produced from *Scenedesmus* sp.

DU and LCSF affects the critical aspects of biodiesel such as CN and OS. High branching number and double bonds create lower combustion properties. CN is relevant to productive ignition properties of fuel and it is related to the OS. CN and OS levels are likewise ASTM 6751 standards. The produced lipid from *C. vulgaris* has CN and OS values 48.39 and 4.78, respectively (Chávez-Fuentes et al., 2018). The collected results in the thesis study show the FAME content of the produced lipid from *S. platensis* is suitable for a subjective fuel production.

Table 3.8 Biodiesel potential parameters of produced lipid from *S. platensis*

	According to Method-1	**According to Method-2**	**European Standard (UNE-EN 14214)**	**American Society for Testing and Materials (ASTM 675)**
IV (g I_2/100 g)	114.44±5.01	40.97±1.69	<120	-
CN	48.56±1.86	69.15±3.20	>51	>47
SV	194.81±9.03	170.22±8.07	-	-
DU	104.34±4.93	45.42±1.77	-	-
LCSF	31.67±1.06	11.85±0.46	-	-
OS	3.76±0.11	6.03±0.28	>6	>3

3.6 Comparison of the Biodiesel Quality of the Produced Lipid with the Literature

As long as the need for energy continues, biofuels will remain popular alternatives. An indication of this is that the potentials of different biological resources in biofuel production continue to be explored. Different biological sources such as microalgae, yeasts and bacteria can be used as raw materials in biodiesel production. While the need for energy is increasing, the damage caused by fossil fuels to the environment is also known. In addition to being the solution to the energy crisis, biofuels are promising for solving critical environmental problems such as high CO_2 emissions and greenhouse gas accumulation. For this reason, the research of the biofuel potential of different biological resources continues. The suitability of lipid produced from different biological sources for the production of high-quality biodiesel was compared with the lipid produced in this study. When the FAME compositions of the lipids produced are compared, it can be said that they have as high-quality content as the examples in the literature (Table 3.9). In addition, it is seen that the main component of the lipids produced consists of C16-C18 fatty acids, which are the most important in biodiesel production. It is seen that lipid production comparable to the FAME content of the lipid produced from different microbial sources such as cyanobacteria, green microalgae and yeast was achieved in this study.

The lipid produced in this study was compared with the samples in the literature in terms of parameters determined as a measure of the suitability of the lipid for the production of high-quality biodiesel (Table 3.10). Compared to lipid produced from different sources such as microalgae and yeast, we see that the produced lipid from *H. pluvialis* and *S. platensis* are suitable sources for quality biodiesel production. In addition, it was determined that the biodiesel potential parameters of the lipid produced in the study were in accordance with European (UNE-EN 14214) and American (ASTM 6751) standards.

Table 3.9 Comparison of the FAME content of the lipid produced in the study with the examples in the literature

Microorganism	**C14:0 (%)**	**C16:0 (%)**	**C18:0 (%)**	**C18:1 (%)**	**C18:3 (%)**	**Reference**
S. platensis	n.d.	59.30	4.50	n.d.	n.d.	Dehaghani & Pirouzfar, 2018
Chlorella sp.	27.80	11.50	1.50	n.d.	n.d.	Dehaghani & Pirouzfar, 2018
B. braunii	n.d.	25	10	35	15	Sadeghin et al., 2018
R.glutinis	1.52	24.89	3.09	42.15	7.33	Vinarta et al., 2020
Leptolyngbya sp.	6.84	17.68	2.65	26.38	n.d.	Tiwari et al., 2020
H. pluvialis	10.30±0.51	16.50±0.77	28.30±1.28	19.90±1.11	n.d.	This study (Method-2)
S. platensis	n.d.	29.63±1.32	17.77±0.88	25.60±1.21	n.d.	This study (Method-2)

Table 3.10 Comparison of the biodiesel potential parameters of the lipid produced in the study with the examples in literature

Microorganism	**CN**	**IV**	**OS**	**DU**	**LCSF**	**Reference**
Synechocytis sp.	54.96	76.33	7.04	68.39	n.d.	Sivaramakrishnan & Incharoensakdi, 2018
Synechococcus sp.	n.d.	n.d.	n.d.	103.7	5.20	Rós et al., 2013
S. obliquus	54.12	110.37	n.d.	n.d.	n.d.	El-Sheekh et al., 2018
C. vulgaris	48	20-100	4.78	77.72	49	Chávez -Fuentes et al., 2018
R. glutinis	51.75	94.18	7.11	96.75	4.03	Viñarta et al., 2020
Leptolyngbya sp.	52.16	84.08	n.d.	84.04	3.09	Tiwari et al., 2020
H. pluvialis	67.03±2.89	34.01±1.66	6.32±0.27	37.70±1.81	25.80±1.26	This study(Method-2)
S. platensis	69.15±3.20	40.97±1.69	6.03±0.28	104.34±4.93	31.67±1.06	This study(Method-2)
STANDARDS	>51	<120	>6	-	-	UNE-EN 14214
	>47	-	>3	-	-	ASTM 675

CHAPTER FOUR
CONCLUSION

Increased use of renewable energy resources by designing tight regulations and long-term planning is one of the upcoming projects inTurkey. In our time most widely used renewable energy sources are wind, solar and hydroelectric power plants. However, use of bioenergy is less preferred. While evaluating the bioenergy potential, domestic wastes, sewage wastes, agricultural and forestry wastes are taken into account, but no evaluation is made for bioenergy production from microbial biomass. Microalgal biofuel production is preferred around the world for reasons such as high efficiency, low space requirement, not being dependent on the season, and rapid production. For these reasons, it is important to reveal the potential of microalgal biofuel production in Turkey.

Biodiesel production from microalgae lipid is a widely studied topic as the production of biodiesel from microalgae is still ambiguous. Biodiesel has several advantages over other biofuels. For example, the production of other liquid biofuels, bioethanol and biobuthanol, requires an anaerobic environment and it is difficult to create these ambient conditions. Since biogas and biohydrogen are in gaseous form, they create difficulties especially in storage and transfer stages. Biodiesel, on the other hand, is a more often used fuel type due to its ease of production, storage conditions and compatibility with existing engines.

S. platensis and *H. pluvialis* are widely studied microalgae for protein, carotenoid and astaxanthin production. Besides lipid production capacity and biodiesel potential of these two microalgae has not been sufficiently investigated. In this thesis study *S. platensis* and *H. pluvialis* were growth in different trophic cultures (photoautotrophic, mixotrophic and heterotrophic) with effect of various environmental and nutritional conditions. The growth characteristics (OD, chlorophyll and protein content) and lipid production potential were investigated. In addition, the fatty acid profile of the produced lipid was determined and its suitability for high quality biodiesel production was examined.

Various industrial wastes such as glycerol and whey have been evaluated as organic carbon sources in mixotrophic and heterotrophic cultures. With this strategy, the cost of the growth medium is reduced as well as contributing to the zero-waste target. For example, in the biodiesel production process, glycerol is released up to 10% by weight of the total product. The use of this waste glycerol as an organic carbon source in the microalgae growth medium and the production of microalgal lipid will support the zero-waste target. Similarly, using whey, which is a waste of approximately 80% by volume of milk used in cheese production, in a microalgal growth environment and obtaining a value-added product is an environmentally friendly and economical approach.

Both of two microalgae, *H. pluvialis* and *S. platensis*, were adapted to mixotrophic conditions and the highest lipid production was detected in the medium which contained crude glycerol as external carbon source. While it is difficult for a photoautotrophic microorganism to acclimate to a growth medium containing a pure carbon source, it is much more ambitious to adapt to a medium containing a waste containing different components. All microalgae cells can not suit in mixotrophic and chiefly heterotrophic cultivations. Chavoshi and Shariati (2019) incubated *Dunaliella salina* in autotrophic, mixotrophic and heterotrophic cultures with glucose or acetate as organic carbon source. The researchers stated that *D. salina* showed low growth rate in these mixotrophic and heterotrophic cultures and lipid production was not detected.

The suitable carbon source for both microalgae was determined as crude glycerol. The compatibility of glycerol with the cell structure and the ease of its transfer into the cell, and the fact that it is quickly and easily metabolized after being taken into the cell are among the main reasons for this situation. It is seen as an important advantage of crude glycerol that it contains methanol at a lower rate than technical glycerol. The reason why the highest lipid productions are obtained in the mixotrophic medium is that both heterotrophic and photoautotrophic metabolism are actively working in this trophic culture. Excess acetyl CoA and energy produced by uptake of both organic and inorganic carbon are directed to TAG production.

After the cultivation type and carbon source were determined, the effects of nitrogen, phosphorus concentrations and light intensity on lipid production were investigated. For *S. platensis* the optimum condition was detected as mixotrophic culture which contains 10 mM crude glycerol, 29 mM nitrogen, 1.5 mM phosphorus and illuminated with 1500 lux light intensity. For *H. pluvialis* the optimum condition was detected as mixotrophic culture which contains 2.5 mM crude glycerol, 2.9 mM nitrogen, 5.6 mM phosphorus and illuminated with 1500 lux light intensity. In the optimum conditions *H. pluvialis* produced 12 times more and *S. platensis* produced 19.17% higher lipid compared to the control condition (photoautotrophic culture). The highest lipid production amount was 5.78±0.21 mg/ g cell from *S. platensis* culture and 11.49±0.57 mg/ g cell from *H. pluvialis* culture.

The lipid produced under optimum conditions was extracted by two different methods. The first of these methods is the most preferred base catalyst method. The second method is the direct FAME production method. The two methods were compared and it was determined that the direct FAME production method was more suitable. The produced lipids from both microalgae in optimum conditions were evaluated as biodiesel source. Produced under optimum conditions, *H. pluvialis* lipid contains 73.60% of C16-C18 fatty acids, which are the most basic for biodiesel. This value was determined as 82.91% for *S. platensis*. For the produced lipid from *H. pluvialis*, saturated fatty acid content is 65.20±2.92%, polyunsaturated fatty acid content is 8.90±0.31% and monounsaturated fatty acid content is 19.90±1.11%. For the produced lipid from *S. platensis*, saturated fatty acid content is 47.40±2.20%, polyunsaturated fatty acid content is 9.91±0.47% and monounsaturated fatty acid content is 25.60±1.21%. Especially the high content of polyunsaturated fatty acids allows the use of the produced lipid in the food industry.

In addition, it was determined that the lipids produced were compatible with the quality biodiesel parameters determined by the relevant associations in Europe and America. CN, IV and OS values of the produced lipid from *H. pluvialis* were detected as 67.03±2.89, 34.01±1.66 and 6.32±0.27, respectively. These values were detected as 69.15±3.20, 40.97±1.69 and 6.03±0.28, respectively for the produced lipid from *S. platensis.*

In terms of compliance with the standards, biodiesel produced from two different types of microalgae is suitable for active use. Nevertheless *H. pluvialis* was more appropriate for lipid production because of its high production yield, wide FAME content scale and compliant with standards biodiesel potential parameters. It is also an advantage for the use of a lower amount of external carbon source in the production medium, short incubation period and the triggering of the production with a nutritional variable such as the amount of phosphorus. The study has shown that, th studied method can be used in evaluation of different industrial wastes in obtaining value-added products even though it is not large scale. The capability of optimum conditions for economic and ecological large scale production can be evaluated.

S. platensis is a widely studied microalgae for protein production. Besides, lipid production capacity and biodiesel potential of this microalgae have not been sufficiently investigated. In terms of compliance with the standards, biodiesel produced from *S. platensis* is appropriate for active use. Due to the high PUFA level, the generated lipid could be assessed as a human or animal safe dietary supplement. *S. platensis* contains polyunsaturated fatty acids, which have a significant role in starvation, raises the starvation characteristics of *S. platensis* in addition to protein content.

Moreover, other beneficial materials returned after the extraction of total lipid to biodiesel can be used in different areas such as in animal nutrition and agriculture. Use of produced cell in multiple areas makes the production more economiclly and ecologically more advantagesous and rewarding (Ferreira, Pinto, Maciel Filho & Fregolente, 2019). Thus, it is also favorable to be able to be generated in various value-added products besides biodiesel using *S. platensis* or *H. pluvialis.* This approach is also in line with the zero-waste target. Remaining cell residue after lipid production can be used in the production of different products, while at the same time they can be used as a source of carbon or nitrogen in a new fermentation.

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