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EFFECTS OF NUTRIENTS ON LIPIDS AND BIODIESEL PRODUCTION POTENTIAL OF *Haematococcus pluvialis*

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Abstract

Biofuels are important energy source alternatives because of their low input and high output properties. *Haematococcus pluvialis* is a freshwater green microalga that is considered as third-generation biofuel feedstock. Production medium conditions and nutritional factors affect the productivity of process and quality of produced biofuel. In this study the effects of carbon sources type (crude or technical glycerol) and concentration (1, 2.5 and 10 mM), nitrogen (0, 1.5 and 2.9 mM) and phosphorus (0, 2.5 and 5.6 mM) concentrations on production of *H. pluvialis* biomass, chlorophyll, and total lipid were investigated. Additionally, the fatty acid profile was detected by gas chromatography and the biodiesel potential was examined. The highest lipid production (11.49±0.57 mg/ g wet cell) was detected with 2.5 mM crude glycerol, 2.9 mM nitrogen and 5.6 mM phosphorus on 5th day of incubation. FAME composition of produced lipid was determined and saturated, polyunsaturated and monounsaturated fatty acid contents were detected as 65.20±2.89%, 8.90±0.31% and 19.90±1.11%, respectively. All these results indicate that *H. pluvialis* has the capability to utilize crude glycerol for lipid production with high phosphorus and sufficient nitrogen and organic carbon concentrations. Additionally, produced lipid might be used as a potential feedstock for a quality biodiesel production due to its fuel characteristics (IV, 34.01±1.66 and CN, 262.44±12.48) according to European Standard.

Key words: biodiesel, glycerol, *Haematococcus pluvialis*, nitrogen, phosphorus

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1. Introduction

The fossil fuel reservoir will be exhausted away in the near future. Prevalent use of petroleum causes the production and increasing concentration of harmful gases (Kim et al., 2019). Biofuels are the important alternatives to fossil fuels with their clean energy, high energy capacity, renewability and biodegradability (Neag et al., 2019). At present, most popular biofuels are biodiesel and bioethanol. Biodiesel is produced generally from vegetable oil which has problems such as low yield, huge land requirement, long production period necessity, susceptibility to seasonal and climatic variations (Poontawee et al., 2018). Microalgae is an alternative feedstock for biodiesel production due to their short doubling time, high adaptation ability, small production area need (Blaga et al., 2018; Yin et al., 2020).

The freshwater microalga *Haematococcus pluvialis* is a promising source for biodiesel production with its lipid content (20-45%) (Damiani et al., 2010). The production of *H. pluvialis* is occurred mainly photoautotropically. While photoautotrophic cultivation is widely used in the laboratory, pilot and industrial scale, microalgae cultivation can be carried out in heterotrophic metabolism, which involves an organic carbon source (Wen et al., 2019). In heterotrophic cultivation, microalgae cells are cultivated in the presence of organic carbon source

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(acetate, glucose, sucrose etc.) but no light (Meireles et al., 2017). The cultivation in which both inorganic and organic carbon sources are present are denominate mixotrophic cultivations. In mixotrophic cultivation organic carbon source type and concentration are important factors on production. The advantages of mixotrophic cultivation are higher growth rate, over biomass and lipid accumulation, maintain of pigmentation production, decline production of CO_2 while there are some issues such as higher cost because of organic carbon source, microbial contamination risk, and reduced energy conversion efficiency (Wang et al., 2017; Zhan et al., 2017). Besides that, the cost of carbon source also affects the choosing of substrate. Crude glycerol is a promising carbon source for microalgal production due to its availability, low economic value and reduced cost (Poontawee et al., 2018). During biodiesel production process approximately 10% (w/w) crude glycerol is produced that has numerous impurities such as methanol, salt, soap (Chol et al., 2018). For this reason, purification of crude glycerol needs high cost and additionally discharging it as waste can cause several environmental problems (Poontawee et al., 2018). Using crude glycerol as carbon source for microalgae growth it provides low-cost production medium and a solution to an environmental problem. Glycerol is nontoxic for microorganisms, it can cross the cytoplasmic membrane through passive diffusion and it can be easily metabolized (Sprenger, 2017).

In addition to carbon source, nitrogen and phosphorus concentrations can affect the lipid production. In generally the lipid production increases in microalgae that is cultivated under nitrogen deficiency (Chakravarty and Mallick, 2019). On the other hand, the increase of phosphorus concentration stimulates lipid production in microalgae (Chakravarty and Mallick, 2019). Several studies reported that microalgae cells can modify their metabolism to increase lipid accumulation under different nutritional conditions (El-Din, 2019; Tosuner and Öztürk Ürek, 2020). The storage lipid has energy storage function that can support the cell to unexpected conditions (Chakravarty and Mallick, 2019). In a study of Zhang et al. (2020), the influences of glycerol on the biomass and lipid accumulation in *H. pluvialis* under low light were investigated. They reported that addition of 1 mL/L glycerol enhanced total lipid substance by 36.8%. Nitrogen starvation effect on *H. pluvialis* lipid production was investigated and nitrogen deprivation activated an intense rise in the content of TAG (Zhekisheva et al., 2002).

Since studies with *H. pluvialis* are limited, this work is focused on the effect of nutrient composition on lipid production of *H. pluvialis.* Effects of carbon sources type and concentration, nitrogen and phosphorus concentrations on production of biomass, chlorophyll, and total lipid were investigated depending incubation period. In addition, lipid extraction was performed after determining the lipid peroxidation and proline content in the conditions where the highest lipid production was obtained. Biodiesel potential was demonstrated after determining the fatty acid profile of the extracted lipid by gas chromatography (GC).

2. Material and methods

2.1. Microalga and growth media

The microalga *Haematococcus pluvialis* CCAP 34/12 was provided from Çukurova University, Faculty of Aquaculture, Turkey. For the maintenance of microalgae under photoautotrophic culture, it has been grown in Bold Basal Medium (BBM) (Janet et al., 1973).

2.2. *Mixotrophic cultivation*

Mixotrophic culture was carried out in BBM which contained different concentrations of crude glycerol (1, 2.5 and 10 mM) or technical glycerol (1, 2.5 and 10 mM) as carbon source. Batch cultivation was carried out in 250 mL Erlenmeyer with 100 mL working volume at 1500 lux (20.25 µmol photon m^{-2} s^{-2}) light intensity (by white fluorescent lamps with continuous illumination), 100 rpm, pH 7.0 and 28°C.

2.3. *Effects of nitrogen and phosphorus concentrations*

After determining the carbon source and concentration for maximum lipid production, the effects of different concentrations (0, 1.5 and 2.9 (BBM concentration) mM) of nitrogen on lipid production of *H. pluvialis* were evaluated in batch cultivation as described above. Then after determining the nitrogen concentration for maximum lipid production, the effects of phosphorus concentration (0, 2.5 (BBM concentration) and 5.6 mM) were studied in the same cultivation conditions.

2.4. *Determination of chlorophyll content*

Chlorophyll a and b contents were measured as described by Lichtenthaler and Wellburn (1983) and calculated using the Eqs. (1) and (2), respectively.

$$Chl\ a = 13.36\ x\ Abs\ 664.2 - 5.19\ x\ Abs\ 648.6 \quad (1)$$

$$Chl\ b = 27.43\ x\ Abs\ 648.6 - 8.12\ x\ Abs\ 664.2 \quad (2)$$

2.5. *Determination of total lipid content*

Total lipid content of microalgae was determined by Mishra et al. (2014) method. The absorbance values of samples at 530 nm were detected.

2.6. *Determination of total carbohydrate content*

The production medium's total carbohydrate content was detected (at 470 nm) according to Dubois

et al. (1956) method.

2.7. *Determination of total protein content*

The cell's protein content was determined (at 595 nm) by Bradford (1976) method.

2.8. *Determination of proline content*

The cell's proline value was determined (at 520 nm) by Bates et al. (1973) method.

2.9. *Determination of lipid peroxidation (LPO)*

The cell's LPO value was determined (at 532 and 600 nm) by Ohkawa eta al. (1979) method. Malondialdehyde (MDA) content was determined according to Eq. (3).

$$MDA = \frac{(A532-A600)\ x\ volume\ of\ extraction}{155\ x\ g\ sample} \quad (3)$$

2.10. Fatty acid methyl ester (FAME) determination

Two different methods have been tried for extraction of lipid and FAME production. In the first method, the cells collected by centrifugation were mixed with 0.8 mL of purified water, 2 mL of methanol and 1 mL of chloroform until a single phase was obtained, followed by addition of 2 mL of purified water and 2 mL of chloroform to obtain a biphasic mixture (Mohammad Mirzaie et al., 2016). After dissolving 100 mg of the extracted dry lipid in 10 mL of hexane, 100 µL of 2 N KOH added in methanol and mixed with vortex for 30 seconds. GC (Agilent 7890 GC, 30 m capillary column, FID) was used to determine the fatty acid content of produced lipid.

In the second method, the cells collected by centrifugation were lyophilized. Direct FAME production was performed using dry cell (Indarti et al., 2005). 15 mg lyophilized cell and 3 mL solution (methanol:sulfuric acid: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 resulting lipid was determined by GC after adding 1 mL hexane to dried lipid.

Flame ionization detector (FID) and 60 m x 0.25 mm capillary column were used for FAME analysis. The flow rate of Helium gas used as carrier gas was adjusted to 1.3 mL/min. The injector temperature was set at 250°C and the detector temperature was set at 270°C. The oven temperature was gradually adjusted to 175°C for 10 min, then to 3°C/min to reach 220°C and remain at that temperature for 5 min. Sigma FAME MIX (C14-C22) fatty acid mixture was used as standard.

2.11. Biodiesel potential of produced FAME

Eqs. (4-9) were used to determine the biofuel production potential of the produced lipid (Rós et al., 2013).

Iodine value:

$$\mathrm{IV} = \frac{\sum(254\ \mathrm{x\ D\ x\ N})}{\mathrm{M}} \quad (4)$$

where: *D* is the number of double bonds; *M* is molecular weight; *N* is the percentage of each fatty acid component).

Cetane number:

$$\mathrm{CN} = 46.3 + \frac{5458}{\mathrm{SV}} - (0.224\ \mathrm{x\ IV}) \quad (5)$$

Saponification value:

$$\mathrm{SV} = \frac{\sum(560\ \mathrm{x\ N})}{\mathrm{M}} \quad (6)$$

Degree of unsaturation:

$$\mathrm{DU} = (\%\ \mathrm{Monounsaturated\ fat\ weight}) + 2\ \mathrm{x}\ (\%\ \mathrm{polyunsaturated\ fat\ weight}) \quad (7)$$

Long chain saturated factor:

$$\mathrm{LCSF} = (0{,}1\ \mathrm{x\ C16}) + (0{,}5\ \mathrm{x\ C18}) + (1\ \mathrm{x\ C20}) + (1{,}5\ \mathrm{x\ C22}) + (2\ \mathrm{x\ C24}) \quad (8)$$

Oxidative stability:

$$\mathrm{OS} = -(0.0384 \times \mathrm{DU}) + 7.77 \quad (9)$$

2.12. Statistical analysis

All analysis were performed and repeated three times (n=3). The final result was obtained by the average of three replicates. Results include standard deviation values. One-way ANOVA test was used to detect the significantly different groups at ($p<0.05$) (SPSS 21.00, USA). Tukey's HSD test was also used to analyze difference among multiple variables. Comparisons were also made with Pearson's correlation.

3. Results and discussion

The high photosynthetic speeds of microalgae authorize them concurrently assemble lipid, carbohydrate and proteins. In the first step of the study the effects of external carbon source type and concentration on lipid production of *H. pluvialis* were investigated depending incubation period. Crude glycerol or technical glycerol was used as organic carbon source in mixotrophic production medium. In terms of total lipid content, the results are given in Fig. 1. The maximum lipid production (4.32±0.22 mg/g cells) was determined in the medium containing 2.5 mM crude glycerol on 14th day of incubation ($p<0.05$). This value is approximately 72% higher than the highest value obtained in technical glycerol-containing media ($p<0.05$). In addition, a higher increase value was obtained from the increase in lipid

production obtained with glycerol by Zhang et al. (2020). This is an indication that *H. pluvialis* adapts to the crude glycerol-containing medium and activates both photoautotrophic and heterotrophic metabolisms. Although there are different findings about the effects of photosynthetic and heterotrophic pathways on each other, this situation varies according to the microorganism (Grama et al., 2016; Noctor et al., 2004). In a study of Grama et al., (2016), utilization of an organic carbon source such as glycerol may affect the relative carbon fluxes in photosynthesis and respiration of *Dactyloroccus dissociates* MT1 .

In the technical glycerol-containing medium, the amount of lipid produced remains low 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. (Choi et al., 2011; Kotzabasis et al., 1999). Similarly, 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.

It can be said that mixotrophic metabolism is caused by accelerating anabolism due to ATP produced in both photochemical and heterotrophic reactions (Kong et al., 2013). One of the reasons why the best result was obtained in the presence of crude glycerol in the mixotrophic cultivation is the ability to improve the transfer system by which the glycerol is introduced into the cell by *H. pluvialis* and the easy metabolization of glycerol taken for the cell (Morales-Sánchez et al., 2015). Once the glycerol is introduced into the cell, it is first phosphorylated with ATP to convert it to glycerol-3 phosphate, followed by glycerol-3 phosphate dehydrogenase to glyceraldehyde-3 phosphate. It is then converted to pyruvate and acetyl Co-A to participate in the TCA cycle (Kong et al., 2013). The other reason may be the microalga prefer for using photosynthesis as an energy source and an organic substrate only as a carbon source such as glycerol (Oliveira et al., 2021).

The changes in total protein and total carbohydrate in the medium where the maximum total lipid value was determined (2.5 mM crude glycerol containing medium) were also investigated (Fig. 2). It is observed that all two metabolite levels first increase and then decrease and remain at a certain level.

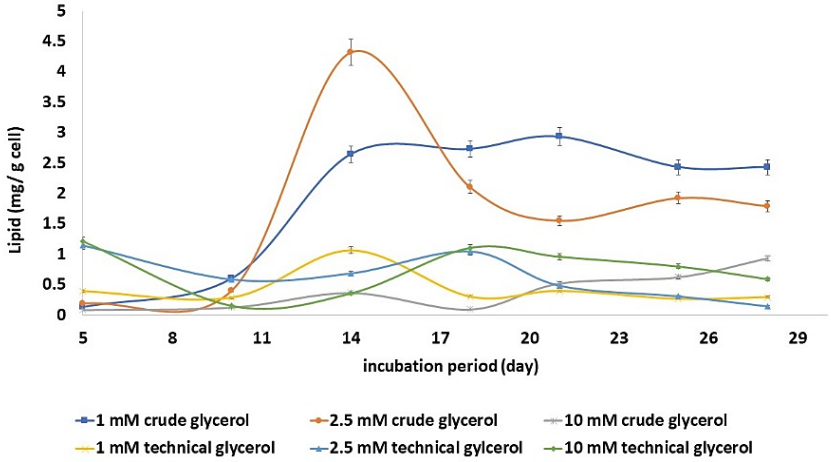

Fig. 1. Total lipid contents of *H. pluvialis* cell with different concentrations of crude or technical glycerol

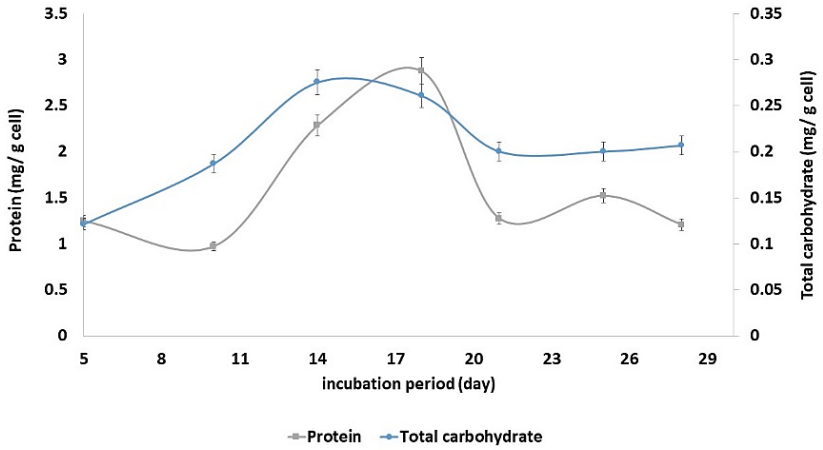

Fig. 2. Protein and total carbohydrate contents of *H. pluvialis* according to incubation period in 2.5 mM crude glycerol containing medium

It can be said that *H. pluvialis* grown in medium containing 2.5 mM crude glycerol tries to adapt to the environment in the first days of incubation. During the adaptation phase, the photosynthesis pathway of the microalga is expected to be active. In photosynthetic pathways, CO_2 is converted to glycerate-3-phosphate to synthesize compounds with storage material such as carbohydrate (Morales-Sánchez et al., 2015). When the microalga is exposed to environmental or nutritional stress and the conditions are not provided for photosynthesis, lipid production occurs as the cell's self-protection mechanism (Fernandes et al., 2013; Narayan et al., 2005). The lipid biosynthetic pathway begins by converting glycerate-3-phosphate to pyruvate and then to acetyl Co-A. The results showed that *H. pluvialis* increased the carbohydrate and lipid deposition by actively stimulating the photoautotrophic metabolism in the first days of incubation. In the following days, it was determined that the biomass increased with the increase of heterotrophic metabolism and the storage material content remained at a certain value.

Chlorophyll a and b levels in the same medium were observed to follow a similar trend while chlorophyll-a value was higher ($p<0.05$) (Fig. 3). Although the light irradiation has not changed during incubation period, the cells were in light-replete condition because of low cell density in the first days of incubation. The maximum chlorophyll a and b values were detected as 74.53±3.24 and 50.20±2.47 mg/g cell, respectively. Due to possible photoacclimation, which occurs by synthesis or breakdown of photosynthetic apparatus in response of variations of photon flux density (MacIntyre et al., 2002; Oliveira et al., 2020), low amount of both chlorophyll pigments in the first days of incubation were detected. The low amount of chlorophyll may be a sign of the photon molecules are accessible for all cells in the culture (Oliveira et al., 2020). In the first days of incubation, it is thought that it tries to adapt to the mixotrophic medium. In addition, increased total carbohydrate and total lipid levels are indicative of accumulation of storage metabolites (Fig. 2). After reaching the highest chlorophyll values, chlorophyll levels decreased in the following days of incubation. It is an indication that *H. pluvialis* adapts to the mixotrophic culture and assimilates the raw glycerol, which is present as a ready carbon source, and therefore reduces its photoautotrophic metabolism and increases its heterotrophic metabolism. There are few studies using glycerol in the growth medium of *H. pluvialis*. In a study sugarcane molasses was used as carbon source in the mixotrophic culture of *H. pluvialis* (Sipaúba-Tavares et al., 2020). The results show that biomass and protein levels were higher than photoautotrophic conditions while produced lipid was decreased. This indicates that each carbon source does not increase lipid production, although it positively affects biomass growth.

After it was determined that *H. pluvialis* reached the maximum total lipid content in medium containing 2.5 mM crude glycerol, the effects of variations in nitrogen concentration on lipid production were investigated. It was found that in the absence and lack of nitrogen, it remained constant in the first days of incubation but increased in the following days (Fig. 4A). 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 phosphorus concentration, lipid production, lipid and carbohydrate content ($p>0.05$). There was a moderate positive correlation between protein and lipid production ($R=0.6471$). The low nitrogen concentration in the environment triggers lipid production as it increases the C/N ratio (Goncalves et al., 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 (Rawsthorne, 2002).

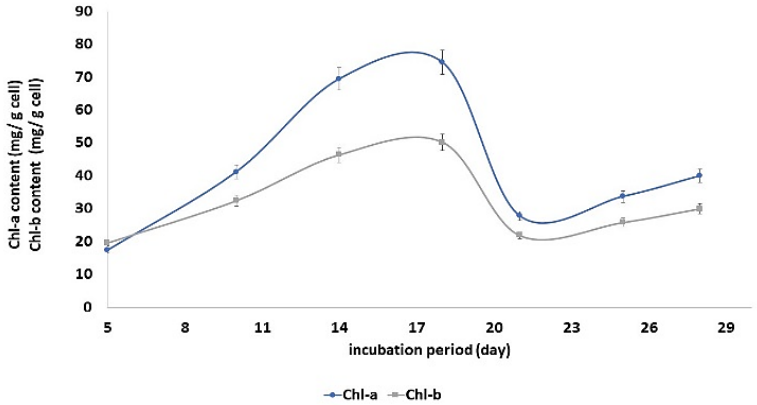

Fig. 3. Chlorophyll contents of *H. pluvialis* according to incubation period in 2.5 mM crude glycerol containing medium

In this respect, although it is thought that the conditions without nitrogen in the production medium were more suitable for lipid production, it was determined that the production medium containing 2.9 mM nitrogen was more suitable because of low biomass values. At high nitrogen concentrations, the total protein content is also expected to be high (Fig. 4B). In line with this expectation, total protein content increased with higher nitrogen concentration. In addition to this total carbohydrate content is increased with rising nitrogen concentration, in parallel with protein content (Fig. 4C). The maximum total carbohydrate and protein contents were detected as 0.28±0.01 mg/g cell and 2.89 ± 0.11 mg/g cell, respectively. There are several studies that investigate the effects of glycerol on lipid production of microalgae (Kong et al., 2013; Poontawee et al., 2018; Sivaramakrishnan and Incharoensakdi, 2018; Zhang et al., 2020). Nitrogen is an essential component of all structural and functional proteins and nucleic acids in cells. Inorganic nitrogen taken up into the cell is promptly assimilated into biochemically active compounds and transferred to the required locations within the cell to meet changing physiological needs. The maximum lipid value was detected in the medium containing 2.9 mM nitrogen ($p<0.05$).

After determining the carbon source type and concentration and the optimum conditions of nitrogen concentration for lipid production of *H. pluvialis*, the effect of phosphorus concentration was investigated depending incubation period (Fig. 5). The highest lipid production was detected as 11.49±0.57 mg/g cell in the presence of 5.6 mM phosphorus concentration on 5th day of incubation ($p<0.05$). This value is approximately 2.66-fold higher than non-optimized conditions (2.5 mM crude glycerol, 2.9 mM nitrogen and 2.5 mM phosphorus containing medium) ($p<0.05$). Additionally, in this optimized condition the lipid production process became faster about 9 days.

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 is involved in many important bioprocesses such as energy transfer, signal transduction, photosynthesis and respiration (Liang et al., 2013).

The amount of produced lipid (11.49±0.57 mg/g cell) is about 92% greater than that produced in photoautotrophic cultivation (data not shown) and about 77% greater than that produced under non-optimized mixotrophic conditions ($p<0.05$). The interaction between phosphorus concentration and lipid production is species-specific (Yu et al., 2019). The results show that high phosphorus concentration stimulates lipid production.

Under environmental stress conditions, photosynthetic electron transport chain accumulates exubarence electrones and these electrones can be used in the production of reactive oxygen species. About 24 NADPH is used for the production of C18 fatty acid. This NADPH is met from the electron transport chain and thus it rests the chain (Wu et al., 2015). Therefore, the cell may be protected by lipid production from the stress condition caused by high phosphorus concentration. 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.

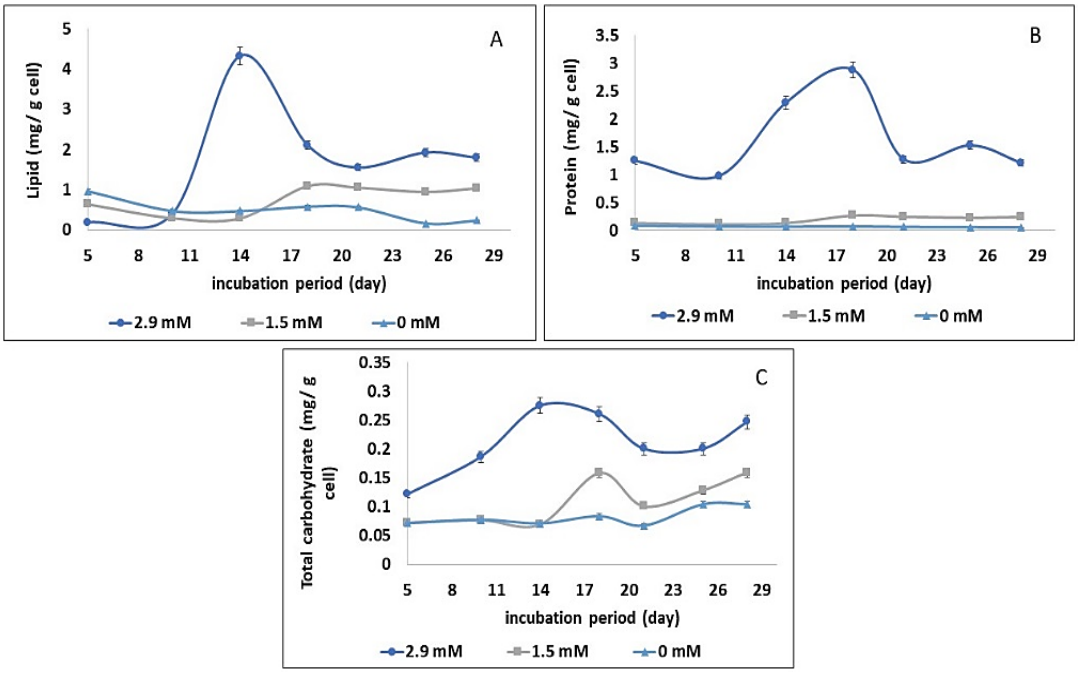

Fig. 4. Lipid (A), protein (B) and total carbohydrate (C) contents of *H. pluvialis* according to incubation period in 2.5 mM crude glycerol containing medium with different nitrogen concentrations

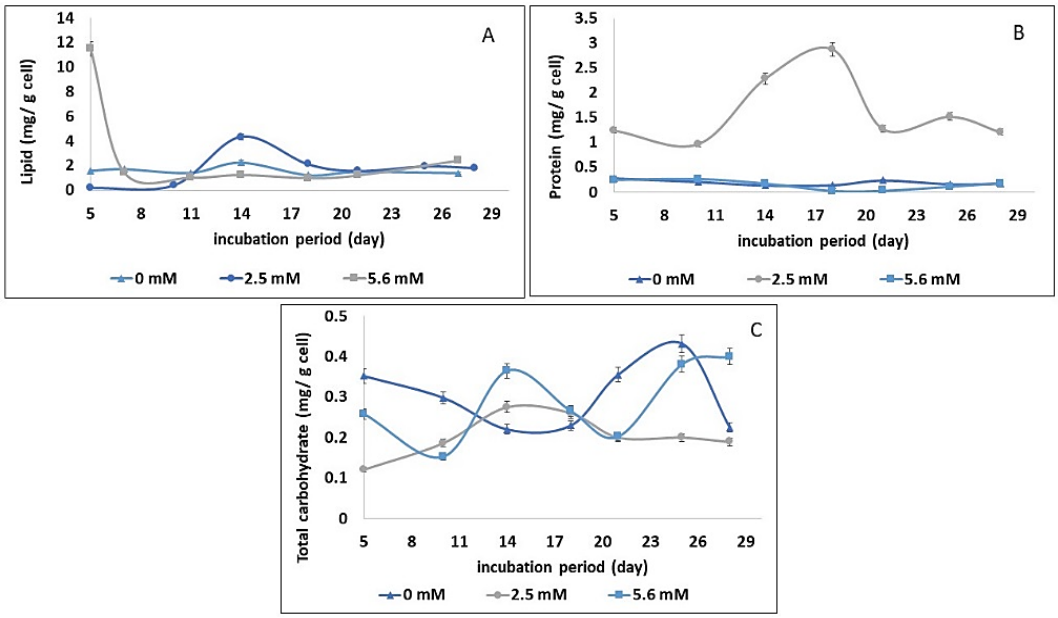

Fig. 5. Lipid (A), protein (B) and total carbohydrate (C) contents of *H. pluvialis* in 2.5 mM crude glycerol and 2.9 mM nitrogen containing medium with different phosphorus concentrations according to incubation period

Thus, it can direct its energy and existing carbon skeleton to lipid production.

Under various nutritional or environmental stresses, microalgae cells change their LPO and proline 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 et al., 2012). Consistent with our study, Bilbao et al. (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 and Ozturk Urek, 2015; Urek and 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 concentration of phosphorus in the growth medium decreases, cell division stops but cell growth continues (Wu et al., 2015). This may be the reason why low protein values are 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 (Fig. 5C).

The excess carbohydrate produced under these conditions acts as structural components of the cell wall (Markou et al., 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 (Fig. 5B) (Markou et al., 2012). The Tukey's HSD test shows there is no meaningful difference among carbohydrate, lipid and phosphoruz concentrations ($p>0.05$). There was a moderate positive correlation between protein and lipid production ($r=0.5513$).

The effect of various nutritional variables was examined FAME composition of produced lipid was determined by GC (Table 1). According to Method 1, saturated fatty acid content is 55.67±2.50%, polyunsaturated fatty acid content is 20.64±0.66% and monounsaturated fatty acid content is 6.48±0.21%. According to Method 2, saturated fatty acid content is 65.20±2.89%, polyunsaturated fatty acid content is 8.90±0.31% and monounsaturated fatty acid 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 et al., 2018). Therefore, the degree of unsaturation and the number of carbons is 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.

Table 1. FAME composition of produced lipid in optimum condition

Type of Fatty Acid	*Percentage of Fatty Acid (Method-1)*	*Percentage of Fatty Acid (Method-2)*
C20:0	ND	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	ND
C18:1n9t	6.48±0.21	19.90±1.11
C18:3	6.31±0.21	ND
C18:2n6c	6.03±0.18	ND
C14:0	4.89±0.13	10.30±0.51

Table 2. Biodiesel potential parameters of produced lipid

	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	262.44±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

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 *Spirulina platensis* microalgae produced fatty acid profile were examined (Dehaghani and 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. were 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 can be said that the fatty acid profile of the lipid produced in the present study is a potential feedstock for biodiesel production because of fatty acid composition.

Saturated fatty acid content is 65.20±2.89%, polyunsaturated fatty acid content is 8.90±0.31% and monounsaturated fatty acid content is 19.90±1.11% according to Method 2. Therefore, the biodiesel potential of the produced lipid was examined. Some parameters such as IV, CN, SV, DU, OS, related to fuel properties of lipid produced under optimized conditions were investigated (Table 2). IV, which shows 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). 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 CN and IV of biodiesel produced from *Scenedesmus obliquus* microalgae was determined as 54.12 and 110.37, respectively and these values were found to be suitable for quality fuel (El-Sheekh et al., 2018). The produced biodiesel from *Synechocystis* sp. grown in the presence of glycerol has an IV of 76.33 and a CN of 54.96 (Sivaramakrishnan and Incharoensakdi, 2018). In another study, *Chlorella 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). In this study the IV of produced lipid was 34.01±1.66, CN was 262.44±12.48 according to Method 2 (Table 2). Similarly, it can be said the lipid produced in this study has suitable properties for high quality fuel production.

All these results indicate that *H. pluvialis* can utilize crude glycerol for lipid production with high phosphorus concentration. Additionally, produced lipid is suitable for a quality biodiesel.

4. Conclusions

Biodiesel production from microalgae lipid is widely studied topic due to the production of biodiesel from microalgae is still ambiguous. This study addressed on the effects of carbon, nitrogen and phosphorus concentrations on lipid production of *H. pluvialis*. The highest lipid production (11.49±0.57 mg/g cell mg) was detected in the presence of 2.5 mM crude glycerol, 2.9 mM nitrogen and 5.6 mM phosphorus concentration on 5th day of incubation ($p<0.05$). Glycerol is a waste with high treatment costs has been transformed into a valuable product by microalga and there is no need for extra treatment processes, and the cost has been reduced since a waste is used in the microalga production medium. The result shows that a higher amount (approximately 12

fold) of lipid can be produced in a mixotrophic culture compared to a photoautotrophic culture without increasing the production cost by using a waste as organic carbon source.

The sufficient nitrogen, excess carbon and phosphorus concentrations directed the *H. pluvialis* metabolism to produce lipids. The fatty acid profile of the produced lipid was analyzed by GC and its biofuel potential was investigated. The IV and CN results (34.01±1.66, 262.44±12.48, respectively) obtained show that the produced lipid is a suitable raw material for a quality fuel according to European standard.

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