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Simultaneous Lipid Production and Valorization of Crude Glycerol by Mixotrophic and Heterotrophic Cultivation of *Arthrospira platensis*

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This study investigates the possible use of crude glycerol as external carbon source for mixotrophic and heterotrophic cultivation of *Arthrospira platensis* for the purpose of producing lipid. Recently, biodiesel has become a remarkable biofuel while cyanobacterial lipid is an alternative feedstock for biodiesel production. In this study effect of carbon source concentration, trophic culture type on production of biomass, chlorophyll, protein, total carbohydrate and lipid were examined. In terms of total lipid content, the highest value was detected as 5.78 ± 0.21 mg/g cell with 10 mM crude glycerol in mixotrophic cultivation on 18th day. Higher specific growth rates were detected in mixotrophic cultures. The fatty acid composition was detected by gas chromatography. The produced lipid had the basic fatty acids for a quality biodiesel production — palmitic, stearic, oleic and linoleic acids. The biodiesel potential of produced lipid was investigated. The indicator of oxidative stability of the fuel, iodine value was in accordance with UNE-EN 14214. Cetane number and oxidative stability are related to productive combustion characteristics of fuel which comply with ASTM 6751 standards. These results show that the fatty acid composition of the produced lipid, from a waste material via economical fermentation, is suitable for a qualitative fuel production.

Keywords: Biofuel potential, Fatty acid composition, Fuel characterization, Glycerol utilization, Heterotrophic culture

Introduction

The cyanobacteria *Arthrospira* sp. has important nutritional properties with high protein, essential amino acid and vitamin content.[1] *Arthrospira* sp. is also a popular cyanobacterium for production of commercially important compounds such as phycocyanin. High adaptability to different environmental conditions, ease of production conditions, synthesis of important biochemical products for food, cosmetics and pharmaceutical industry are the main reasons why this microalgae type continues to be used in different studies.[2]

Production type for cyanobacteria is usually called phototrophic culture.[3] Heterotrophic cultivation is an alternative culture type in which there is an external organic carbon source but no light.[4] Another option for cyanobacteria production is mixotrophic cultivation that contains an organic and inorganic carbon source and also light.[4] In the study of Lai *et al*. (2019), *Arthrospira platensis* was grown on different concentrations of sucrose to produce total carbohydrate.[5] Similarly, in our previous study the biomass, chlorophyll, and total lipid production of *A. platensis* was investigated with mixotrophic cultivation.[6] Heterotrophic and mixotrophic cultures have some advantages over phototrophic cultivation such as higher growth rate, higher biomass, protein, lipid production etc. Despite many advantages of these culture types, there are some problems such as contamination risk and higher cost due to organic carbon source.[7] The cost of carbon source also affects the choice of carbon source type.[8] Crude glycerol, the main by-product from biodiesel production process, is a potential carbon source for microbial fermentation owing to its availability, low commercial value and low cost.[9] As it is suitable with bio molecules it has osmoregulatory characteristic. Due to numerous impurities such as methanol, salt, soap, discharging of crude glycerol as waste is likely to cause several environmental problems. Using such wastes as carbon source for cyanobacteria production provides low cost production medium, value-added products, conserves the energy to be used for waste treatment and a solution to an environmental problem.

Renewable energy sources are one of these value-added products which are now recognized as remarkable sources.[10] Biodiesel has better

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performance than diesel and also it has low hydrocarbon emission.[11] Biodiesel is an important alternative energy fuel with their use in diesel engines without requiring modification.[12] Cyanobacteria and microalgae lipids are alternative and notable sources with fast production time, small production area need, cleaner foot print etc.[12]

Arthrospira sp. has been preferred due to its suitable metabolism to the different trophic cultures in which low-cost carbon sources are used. As studies using glycerol in the mixotrophic or heterotrophic growth medium of *A. platensis* is still limited, in the present study this cyanobacterium was grown in different trophic cultures with crude glycerol as carbon source. Effects of carbon source concentration, trophic culture type on production of biomass, chlorophyll, protein, total carbohydrate and total lipid were investigated. Also two different lipid extraction methods were examined. Additionally, the biodiesel potential of produced lipid was investigated and the fatty acid profile was detected by gas chromatography.

Materials and Methods

Microalgae and Growth Media

The cyanobacteria *Arthrospira platensis* (Gamont) Geitler 1952 was provided by Çukurova University, Faculty of Aquaculture, Turkey. For the sustenance of cyanobacteria under phototrophic culture, it was grown in Zarrouk's Medium (pH 9.0).[13] Batch cultivation was implemented in 750 mL working volume/1 L serum bottle with continuous illumination (2500 lux (33.75 µmol photon $m^{-2} \cdot s^{-2}$) by white fluorescent lamps), at 30°C and the cultures were mixed and aerated using filtered air continuously.

Mixotrophic and Heterotrophic Cultivation

Mixotrophic and heterotrophic cultures were applied in Zarrouk's Medium (pH 9.0) which contained different concentration of crude glycerol (1, 2.5 and 10 mM) as external carbon source. Culture was inoculated to an initial optical density (OD = 600 nm) of 0.2. Since *A. platensis* is a filamentous micro organism, before reading the OD the culture was transferred to spectrophotometer cuvette and the cuvette was turned upside down for three times.[6]

Batch cultivation was operated in 100 mL working volume/250 mL Erlenmeyer flask at 100 rpm, 30°C. For mixotrophic culture continuous illumination (1500 lux or 20.25 µmol photon $m^{-2} \cdot s^{-2}$) was provided by white fluorescent lamps.

Specific growth rate (µ) was calculated according to equation below.

$$\mu = \ln \frac{X1 - X0}{t1 - t0} \quad \ldots (1)$$

(X: amount of microorganism, t: time as day).

Phototrophic cultivation was used as control condition.

Determination of Total Lipid Content

Total lipid content of cyanobacteria was determined by Mishra *et al*. (2014) method.[14] The absorbance was measured at 530 nm against a reference sample.

Determination of Chlorophyll Content

Chlorophyll a contents were measured as described by Lichtenthaler and Wellburn (1983).[(15)] The algal suspension was collected by centrifuged (5000 rpm, 15 min, 4°C) and then homogenized in absolute ethanol by 8000 rpm for 1 min and 9500 rpm for 1 min with 30 seconds intervals.[16] The obtained supernatant (12000 rpm, 10 min, 4°C) was measured at 664.2 and 648.6 nm. Chlorophyll contents were calculated according to Eq. 2.

$$\text{Chl a} = 13.36 \times \text{Abs}_{664.2} - 5.19 \times \text{Abs}_{648.6} \quad \ldots (2)$$

Determination of Protein Content

Collected cells by centrifugation were homogenized with 50 mM, pH 7.0 phosphate buffer, followed by centrifugation (12000 rpm, 10 min, 4°C), and the supernatant was used for the analysis of protein content.[16] Protein quantification was carried out by the Bradford method at 595 nm.[17]

Determination of Total Carbohydrate Content

Total carbohydrate content of cyanobacteria cell was detected by phenol-sulphuric acid method.[18] The absorbance was measured at 470 nm against a reference sample.

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.[19] 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.

In the second method, the cells collected by centrifugation were lyophilized. Direct FAME

production was performed using dry cell[20] 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 resulting lipid was determined by gas chromatography (Agilent 7890 GC, 30 m capillary column, FID, He gas) after adding 1 mL hexane to dried lipid.

Flame ionization detector (FID) and 60 m × 0.25 mm capillary column were used for FAME analysis. The flow rate of He 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.

Biodiesel Potential of Produced FAME

The following formulas were used to determine the biofuel production potential of the lipid produced.[21]

Iodine value

$$IV = \frac{\sum(254 \times D \times N)}{M} \quad \ldots (3)$$

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

Cetane numbe

$$CN = 46.3 + \frac{5458}{SV} - (0.224 \times IV) \quad \ldots (4)$$

Saponification value

$$SV = \frac{\sum(560 \times N)}{M} \quad \ldots (5)$$

Degree of unsaturation:

$$DU = (\%\ \text{Monounsaturated fat weight}) + 2 \times (\%\ \text{polyunsaturated fat weight} \quad \ldots (6)$$

Long chain saturated factor

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad \ldots (7)$$

Oxidative stability

$$OS = -(0.0384 \times DU) + 7.77 \quad \ldots (8)$$

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. The data were analyzed by analysis of variance (TUKEY) to identify the significantly different groups at ($p<0.05$) by one-way TUKEY test using SPSS software statistical program (SPSS for windows ver. 21.00, USA).

Results and Discussion

The cyanobacteria *A. platensis* was incubated under phototrophic, mixotrophic and heterotrophic cultivation conditions, with different concentrations of crude glycerol. In terms of total lipid content, the highest value was detected as 5.78 ± 0.21 mg/g cell with 10 mM crude glycerol in mixotrophic cultivation on 18th day (Fig. 1A) ($p<0.05$). Lipid production in heterotrophic cultures was lower than mixotrophic cultures. Maximum lipid production (2.46 ± 0.10 mg/g cell) in heterotrophic cultures was detected with 10 mM crude glycerol on the 5th day of incubation (Fig. 1B). In mixotrophic culture the lipid value was detected as 0.47 ± 0.19 mg/g cell 10 mM crude glycerol on 5th day. The lipid values were lower with below crude glycerol concentrations in both the culture conditions.

Lipid content of *A. platensis*, which is a popular cyanobacterium about protein contents, was increased with this study. When these results were compared, it was determined that the highest lipid production was obtained in mixotrophic culture ($p<0.05$) (Fig. 1A). The highest lipid production was detected with 10 mM crude glycerol in mixotrophic culture and this value was about 19.17% higher than the control

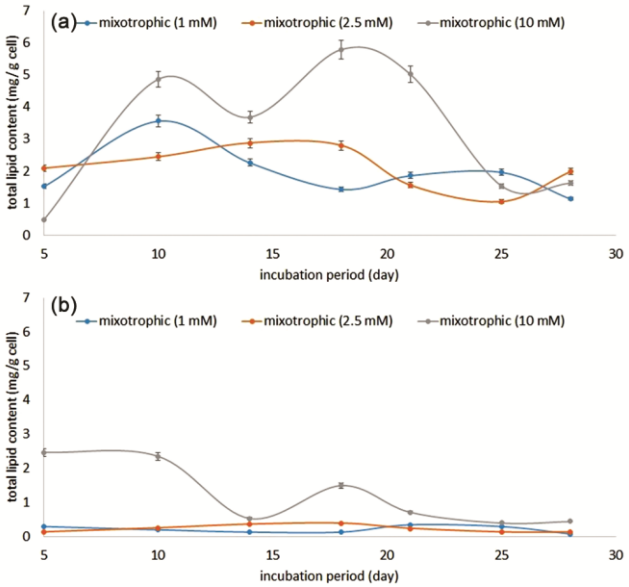

Fig. 1 — Total lipid content of *A. platensis* cell with different concentration of crude glycerol in (A) mixotrophic or (B) heterotrophic cultures. The values are the mean ± SD for experiments of three separate experiments

condition ($p<0.05$). It can be concluded that lipid production is more productive in mixotrophic cultivation in which both metabolisms work together compared to the cultivation types in which only phototrophic metabolism and heterotrophic metabolisms work.[22] 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 phototrophic and heterotrophic metabolisms produce energy and provide carbon skeleton for lipid production. It is known that the excessively produced energy is stored in triacylglycerol (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 (Fig. 1B and 2B).

When the effect of the carbon sources concentration on lipid production was examined, it was determined that 10 mM crude glycerol triggered lipid production more than lower concentrations. The low nitrogen concentration in the medium triggers lipid production as it increases the C/N ratio.[23] 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. 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. Carbohydrate will provide both pyruvate as carbon skeleton and ATP and reducing power (NADPH) required for synthesis for lipid synthesis. Glycerol is inserted into the cell by diffusion, and by phosphorylation with ATP it is converted to glycerol-3 phosphate. Thus, it enters the TCA cycle and supports energy metabolism.[24] In the similar way, the study of Morais *et al*. (2019) showed that the highest lipid productivity occurred with 50 mM glycerol containing medium.[2] With lower glycerol concentrations lipid productivities decreased because of lower C/N ratio.

A. platensis in heterotrophic and mixotrophic cultures, with the highest lipid values, were evaluated for specific growth rates. In heterotrophic culture the specific growth rate was detected as 0.011 day^{-1} with 10 mM crude glycerol. In mixotrophic culture this value was 0.022 day^{-1} with 10 mM crude glycerol. The specific growth rates in mixotrophic cultivation were higher than in heterotrophic cultivations. This situation was supported by protein amounts (Fig. 2). It has been determined by different studies that mixotrophic culture supports growth more than heterotrophic culture.[7] 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.

The highest lipid production media were determined in heterotrophic and mixotrophic cultures and protein, chlorophyll and total carbohydrate levels in these media were investigated (Fig. 2). While the total carbohydrate contents remained low values in both production media the highest chlorophyll-a content (175.98 ± 7.94 mg/g cell) was obtained in the mixotrophic culture as expected (Fig. 2A) ($p<0.05$). The low protein value in heterotrophic culture parallels the low specific growth

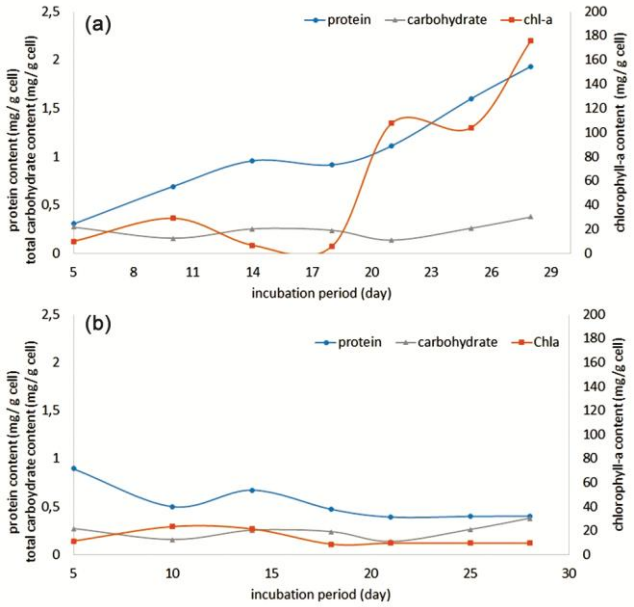

Fig. 2 — Protein, total carbohydrate and chlorophyll-a content of *A. platensis* in 10 mM crude glycerol containing (A) mixotrophic culture, (B) heterotrophic culture. The values are the mean ±SD for experiments of three separate experiments

rate (Fig. 2B). While protein and chlorophyll-a 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 indicates that heterotrophic metabolism is active (Fig. 2A). 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, phototrophic metabolism may have been activated. CO_2, fixed by chlorophyll, in addition to the external carbon source, provides a carbon source that can be used in lipid production.[23] In this 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 Morais *et al*., 2020.[(25)]

With the decrease in the amount of external carbon source, the activity of phototrophic metabolism increased and the amount of chlorophyll increased. In the heterotrophic culture, the amount of chlorophyll decreased during the incubation period (Fig. 2B). This indicates the adaptation process of the inoculum from phototrophic 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, and it appraises its adaptation to environmental stress status.[26] These effects can direct the metabolism to the synthesis of biomolecules.

It was determined that the total amount of carbohydrates did not change significantly during the incubation period in both two cultivations type ($p>0.05$) (Fig. 2). 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.[2] Markou *et al*. (2019) suggest that glycerol has an energy carrier function proteins and lipids synthesis, apart from carbohydrates in the *Spirulina* culture.[27]

The fatty acid composition was detected by extracting lipid from the medium where the highest lipid production occurred (Table 1). According to method-1, saturated fatty acid content is 38.31 ± 0.20%, polyunsaturated fatty acid content is 42.69 ± 2.15% and monounsaturated fatty acid content is 18.96 ± 0.91%. According to method-2, 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%.

The produced lipid from *A. platensis* had the basic fatty acids for a quality biodiesel production, palmitic, stearic, oleic and linoleic acids. Nautiyal *et al*. (2014) studied fatty acid content of *Spirulina* sp. and palmitic, linoleic and linolenic acids determined as the major fatty acid compounds.[28] In the study of Morais *et al*. (2019), *Arthrospira* sp. was cultivated with different concentrations of glycerol.[2] That study shows the main fatty acid composition (89.93%) of *Arthrospira* sp. has 16–18 carbons. Similarly, in this study a significant part (86.03 ± 0.38%) of the total fatty acid content is 16–18 carbons ($p<0.05$). The high degree of unsaturation is important because it regulates membrane fluidity, thermal adaptation, electron and oxygen transport.[29] Palmitic acid (C16:0) is an important content for biodiesel production.[23] Several cyanobacteria and bio energy seeds contain palmitic acid. In this study in method-2 palmitic acid content is higher than method-1. In the study of Dehaghani and Pirouzfar (2018), the produced lipid from *Chlorella* sp. has very low C16 and C18 content which indicates that the produced lipid is not suitable for biodiesel production.[30] Similarly in a study lipid production was occurred from a cyanobacteria type *Leptolyngbya* sp. and the FAME content was analyzed.[31] Especially C18 fatty acid content was detected very low. These data show that the lipid produced in our study is a more suitable biodiesel raw material compared to many studies in the literature.

The biodiesel potential of the produced lipid from *A. platensis* was examined by considering these values (Table 2). According to the FAME composition produced lipid, the degree of unsaturation was 104.34 ± 4.93 and the long chain saturation factor was calculated as 31.67 ± 1.06 in method-1. These values were detected as 45.42 ± 1.77 and 11.85 ± 0.46 in method-2.

Table 1 — FAME composition of produced lipid in mixotrophic culture with 10 mM crude glycerol

Type of Fatty Acid	Percentage of Fatty Acid (method-1)	Percentage of Fatty Acid (method-2)
C22:0	13.96 ± 0.41	ND
C18:3	21.69 ± 1.02	ND
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

ND: not detected

Table 2 — Biodiesel potential parameters of produced lipid in mixotrophic culture with 10 mM crude glycerol

	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	11.8 5± 0.46	—	—
OS	3.76 ± 0.11	6.03 ± 0.28	>6	>3

Saponification value is an index of purity of lipid and for biodiesel production, the lipids with high SV need higher methanol volumes, and generate higher by product.[32] In the present study the SV was detected as 194.81 ± 9.03. Similarly, *Synechocystis* sp. was cultivated with glycerol supplementation and SV of produced lipid was reported as 211.29.[(30)] Iodine value of lipid, the indicator of oxidative stability of the fuel, was in accordance with European Standards (UNE-EN 14214) produced from *A. platensis*. In a study of El-Sheekh *et al*. (2018) lipid production from *Scenedesmus* sp. was carried out and IV of produced lipid was detected as 110.37 g I_2/100 g.[33] The IV value of produced lipid from *Chlorella* sp. was detected in the range of 20−100. This wide range indicates that the stability of the lipid produced is low.[34] This data could be interpreted as the produced lipid in our study is more stable and appropriate source for biodiesel production.

Degree of unsaturation and LCSF affect the important characteristics of biodiesel such as CN and OS. High double bond and branching number cause lower ignition properties. Cetane number is related to productive combustion characteristics of fuel, an indicator of ignition quality of fuels and it is relevant to the OS. Cetane number and OS values of the produced lipid comply with ASTM 6751 standards. In the study of Chávez-Fuentes *et al*. (2018), the produced lipid of *C. vulgaris*, which was cultivated under yellow light, has CN and OS values 48.39 and 4.78, respectively.[34] The obtained results in this present study demonstrate that the fatty acid composition of the produced lipid from *A. platensis* is appropriate for a qualitative fuel production.

All microalgae cells couldn't adapt in mixotrophic and especially heterotrophic cultures. In the study of Chavoshi and Shariati (2019)[(12)] *Dunaliella salina* was incubated in autotrophic, heterotrophic, and mixotrophic conditions with acetate or glucose as carbon source. The researchers declared that the growth rate of the *D. salina* in these conditions showed a sudden decrease and lipid production was not detected. In this present study *A. platensis* was growth in mixotrophic and heterotrophic conditions with crude glycerol as carbon source and remarkable lipid production was detected.

A. platensis is widely studied microalgae for protein production. Besides, lipid production capacity and biodiesel potential of these microalgae have not been sufficiently investigated. In terms of compliance with the standards, biodiesel produced from *A. platensis* is appropriate for active use. The lipid produced is comparable to the studies in the literature in terms of FAME content and biodiesel quality criteria. When the FAME composition and quality standard values are examined, it is seen that the lipid produced in this study is a suitable option for biodiesel production. Due to the high PUFA value, the produced lipid could be also evaluated as a human or animal safe dietary supplement. *Arthrospira platensis* contains poly unsaturated fatty acids, which have an important place in nutrition, increase the nutritional properties in addition to protein content.

Conclusions

In this study an industrial waste was evaluated as carbon source in *A. platensis* growth medium. This study has created both an alternative way of disposal of waste materials and provided a value-added product. Evaluation of the produced cell in production of multiple bio-products makes the process more advantageous in economically and ecologically. Hence, it is also advantageous to be able to produce different value-added materials besides biodiesel using *A. platensis*. Remaining cell debris 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. The study showed that, although it is in laboratory scale, it can be used in the evaluation of various industrial wastes in obtaining value-added products.

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