AQUATIC RESEARCH

E-ISSN 2618-6365

Aquatic Research 3(1), 1-12 (2020) • https://doi.org/10.3153/AR20001 *Research Article*

Evaluation of sucrose as carbon source in mixotrophic culture of *Arthrospira platensis* Gomont 1892

Zülfiye Velioğlu Tosuner[1], Raziye Öztürk Ürek[2]

Cite this article as:

Velioğlu Tosuner, Z., Öztürk Ürek, R. (2020). Evaluation of sucrose as carbon source in mixotrophic culture of *Arthrospira platensis* Gomont 1892. *Aquatic Research*, 3(1), 1-12. https://doi.org/10.3153/AR20001

[1] Dokuz Eylül University, Graduate School of Natural and Applied Sciences, Department of Biotechnology, 35160 Buca, Izmir, Turkey

[2] Dokuz Eylül University, Faculty of Science, Department of Chemistry, 35160 Buca, Izmir, Turkey

ORCID IDs of the author(s):

Z.V.T. 0000-0001-9181-6619

R.Ö.Ü. 0000-0002-7147-6853

Submitted: 07.08.2019

Revision requested: 03.09.2019

Last revision received: 04.10.2019

Accepted: 06.10.2019

Published online: 25.10.2019

Correspondence:

Zülfiye VELİOĞLU TOSUNER

E-mail: zulfiyevelioglu@gmail.com

ABSTRACT

Cyanobacteria are photosynthetic microorganisms that use CO_2 as carbon source and sunlight as energy source. Although phototrophic cultivation is widely used in cyanobacterium production, heterotrophic and mixotrophic cultivations attract attention among researchers. In this study the effect of different concentrations (0[control] - 0.25 - 2.5 - 10 - 50 mM) of sucrose on the growth of *Arthrospira platensis* under mixotrophic cultivation was investigated. The purpose of this study was to investigate whether *A. platensis* biomass production could be performed regardless of high light intensity. Biomass, chlorophyll, lipid and carbohydrate contents were determined by spectrophotometrically. Also the physicochemical properties of the produced cyanobacterium were investigated by FTIR, TGA and DSC. The highest biomass productivity was detected as 1.33 g/L/day in the medium containing 2.5 mM sucrose and the specific growth rate increased 1.32 fold as compared to phototrophic culture. Additionally, the highest lipid content (3.68 ±0.17 mg/g cell) was determined in the same medium. This suggests that *A. platensis* has adapted to the medium that contains low sucrose concentrations. Also, this study showed that sucrose containing medium supports lipid production.

Keywords: Sucrose, Mixotrophic culture, Phototrophic culture, *Arthrospira platensis,* Lipid production

Introduction

Cyanobacteria are photosynthetic microorganisms that use CO_2 as a carbon source and sunlight as energy (Katiyar et al., 2017; Patel et al., 2017). In laboratory scale this natural production type is called phototrophic culture. Although phototrophic cultivation does not need organic carbon source, this culture type makes slow cell growth, low biomass, and higher harvesting cost (Gim et al., 2016; Ozturk Urek & Kerimoglu, 2019). While phototrophic cultivation is widely used in the laboratory, pilot and industrial scale, cyanobacterium cultivation is also performed in heterotrophic cultivation, which contains an external carbon source (Joannesa et al., 2016). In heterotrophic cultivation, cyanobacterium cells are grown in the presence of external carbon source (acetate, glucose, sucrose etc.) but no light (Meireles et al., 2017). The medium in which both CO_2 and an external organic carbon source are present as carbon sources are mixotrophic cultivations. Some microalgae, such as *Chlorella regularis*, *Nannochloropsis* sp., *Synechococcus* sp., *Anabaena* sp., *Arthrospira platensis*, can grow better under mixotrophic condition, which may combine the advantages of phototrophic and heterotrophic cultures (Zhan et al., 2017). The advantages of mixotrophic cultivation are higher growth rate, higher biomass and lipid accumulation, sustain of pigmentation and phytochemicals production, decreased production of CO_2 while there are some problems such as higher cost because of organic carbon source, contamination risk, and reduced energy conversion efficiency (Van Wagenen et al., 2015; Wang et al., 2017; Zhan et al., 2017). When compared with heterotrophic culture, the biomass production in mixotrophic cultivation is not only dependent on the carbon source type and amount. Similarly, in mixotrophic cultivation, there is no need for light intensity as high as in phototrophic cultivation, and light dependence is lower (Abreu et al., 2012). As in mixotrophic growth cyanobacteria showed different metabolic activity from phototrophic culture. Photosynthesis and aerobic respiration are stimulated simultaneously in mixotrophic cultures. Mixotrophic growth offers increasing microbial cell concentration in addition to protein, carbohydrate and lipid productivity. Therefore, mixotrophic cultivation is more economical and easier to control than the other two cultivation types.

In a study growth, lipid and biomass productivity of *Chlorella vulgaris* and *Leptolyngbya* sp. in heterotrophic and mixotrophic regimes were investigated (Silaban et al., 2014). Dextrose and sodium acetate were used as external carbon source and the highest biomass productivity (156 g/m^{3}d) and neutral lipid productivity (24.07 g/m^{3}d) was detected with 2.1 g/L sodium acetate in mixotrophic culture. In a study of Ceron Garcia et al., (2006) *Phaeodaciylum tricornutum* was grown in mixotrophic culture which contains fructose, glucose, mannose, lactose or glycerol as external carbon source. Glycerol (0.1 M) was detected as the best substrate that increased final biomass level by 7 fold relative to control cultures. *Arthrospira platensis* cyanobacterium and *Chlorella homosphaera* microalgae were cultivated with glucose in mixotrophic conditions and resulting in biomass increases of up to 3.45 and 2.79 fold, respectively (Margarites et al., 2017).

Since the cyanobacteria *Arthrospira* sp. has important nutritional properties with high protein, essential amino acid and vitamin content, it is an important fish diet alternative (Rosas et al., 2018; Sivakumar et al., 2018). *Arthrospira* sp. can utilize organic carbon substrates in heterotrophic and mixotrophic conditions (Marquez et al., 1993). The blue-green algae *A. platensis* grows mainly on inorganic carbon source and much work has not been carried out on the utilization of organic carbon sources. Some of monosaccharides and disaccharides such as glucose, fructose, sucrose and lactose have been used for mixotrophic cultivation of cyanobacterium and different transport and assimilation mechanisms may be effective for each sugar (Chojnacka & Marquez-Rocha, 2004).

A. platensis is suitable as a biotreatment material for fish production effluents which shows adaptation in mixotrophic cultures. In a study, *A. platensis* was inoculated to the fish culture effluent in order to remove the dissolved nutrients (Nogueira et al., 2018). The concentration of ammonia, nitrite, nitrate and phosphate was detected lower by more than 94.8%, and maximum *A. platensis* productivity was determined as 0.03 g/L. day.

In the study of Chojnacka and Noworyta (2004), the influence of growth parameters on specific growth rate of *Arthrospira* sp. in photoautotrophic, heterotrophic and mixotrophic batch modes were investigated and the highest specific growth rate (0.055 h^{-1}) was reached in mixotrophic culture with 2.5 g/L glucose.

Some studies have thus focused on finding cheaper organic carbon sources to decrease production cost (Bhatnagar et al., 2011; Lin & Wu, 2015). Sucrose present in the waste of the sugar production process is an important alternative carbon source (Abreu et al., 2012; Wang et al., 2016). The use of sucrose-containing waste as a carbon source will evaluate of a waste material and provide low cost production (Mitra et al., 2012). Therefore, it is important to investigate the growth and production aspects of cyanobacterium in sucrose containing medium.

In this study *A. platensis* was cultivated under mixotrophic cultivation with different concentrations of sucrose as a carbon source. Effects of carbon source's concentration on production of biomass, chlorophyll, and total lipid were investigated. Also specific growth rates were calculated. The aim of this study was to investigate the effect of sucrose concentration on growth and lipid production of *A. platensis.* The lipid production of *A. platensis* in sucrose-containing growth medium was investigated for the first time in this study. Also the characterization of produced cyanobacterium cell with TGA and FTIR is a novel approach.

Material and Methods

Cyanobacteria and Culture Media

The cyanobacteria *Arthrospira platensis* (Gomont) 1892 was provided from Cukurova University, Faculty of Aquaculture, Adana-Turkey. For the maintenance of cyanobacteria under phototrophic culture, it has been growth in Zarrouk's medium (Zarrouk, 1966). Batch cultivation was carried out in 750 mL medium at 2500 lux (33.75 µmol photon m^{-2} s^{-2}) light intensity (by white fluorescent lamps) with continuous illumination, pH 9.0 and 30°C and the cultures were mixed and aerated using filtered air continuously.

Mixotrophic Cultivation

Mixotrophic culture was carried out in Zarrouk's medium, which contained different concentration of sucrose (0 [control] – 0.25 – 2.5 – 10 – 50 mM) as carbon source. Culture was inoculated to an initial optical density (OD) of 0.2 at 600 nm (Vonshak et al., 1982). OD is a parameter used to determine biomass production. When working with filamentous microorganisms, make sure that the culture medium is well mixed before reading the OD. In this present study, well mixed *A. platensis* culture was transferred to spectrophotometer cuvette and the cuvette was turned upside down for three timed and then OD was read.

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 shaking rate (Thermoshake Incubator, Gerhardt, Germany), pH 9.0 and 30°C. OD, pH, chlorophyll and total lipid content were detected during incubation period. Zarrouk's medium without any external carbon sources was used as control condition.

Specific growth rate (µ) and biomass productivity (P) were calculated according to Eq. 1 and 2 based on OD values (Kong et al., 2013) (X: amount of microorganism, t: time as day).

$$\mu = \ln \frac{X1-X0}{t1-t0} \qquad \text{Eq. 1}$$

$$P = \frac{X1-X0}{t1-t0} \qquad \text{Eq. 2}$$

Determination of Chlorophyll Content

Chlorophyll a and b contents were measured as described by Lichtenthaler and Wellburn (1983). 5 mL of algal suspension was centrifuged at 5000 rpm for 15 min. Pellet was weighted and homogenized in 5 mL absolute ethanol by 8000 rpm for 1 min and 9500 rpm for 1 min with 30 seconds intervals with laboratory homogenizer (Ultra Turrax, IKA, Germany). After centrifugation absorbance of the obtained supernatant was measured at 470, 664.2 and 648.6 nm. Chlorophyll a and b contents were calculated according to Eq. 3 and 4 (Lichtenthaler & Wellburn, 1983).

$$\text{Chl a} = 13.36 \times \text{Abs}_{664.2} - 5.19 \times \text{Abs}_{648.6} \qquad \text{Eq. 3}$$

$$\text{Chl b} = 27.43 \times \text{Abs}_{648.6} - 8.12 \times \text{Abs}_{664.2} \qquad \text{Eq. 4}$$

Determination of Total Lipid Content

Total lipid content of cyanobacterium was determined by Mishra et al., (2014) method. To prepare reagent 0.6 g vanillin was dissolved in 10 mL ethanol and mixed 90 mL distilled water and 400 mL concentrated phosphoric acid. 2 mL concentrated sulfuric acid was added to 100 µL cyanobacteria sample and was heated for 10 min at 100°C, and was cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added and the sample was incubated for 15 min at 37°C incubator shaker at 150 rpm. The absorbance was measured at 530 nm against a reference sample.

Determination of Total Carbohydrate Content

Total carbohydrate content of production medium was determined by phenol-sulphuric acid method (Dubois et al., 1956). To determine total carbohydrate content, 1 mL cell free supernatant (1 mL distilled water for reference) was mixed with 1 mL 5% (w/v) phenol solution and 5 mL concentrated H_2SO_4. After well mixing the samples were incubated for 20 min at room temperature the absorbance was measured at 470 nm against a reference sample. Glucose was used as standard in the range of 0- 250 µg/mL.

TGA and FTIR Analysis

TGA and DSC analyses of produced cyanobacterium in phototrophic and mixotrophic cultures were carried out with Perkin Elmer- Diamond TG/DTA (Massachusetts, USA). About 3-5 mg of dry produced cyanobacterium cell sample was loaded on a platinum pan and its energy level was scanned in

the ranges of 30 - 500°C under a nitrogen atmosphere with a temperature gradient of 10°C/min.

To analyze the organic structure of produced *A. platensis* cell, the FT-IR spectra were recorded on the Perkin Elmer Spectrum BX (Massachusetts, USA), in the 4000- 400 cm^{-1} spectral region with deuterated triglycine sulfate detector. All samples were dried at 70°C overnight before analysis. KBr pellet was used as a back ground reference. Approximately 1 mg of the sample was milled with approximately 100 mg of dried KBr and then pressed to form a pellet for measurement.

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 (ANOVA) to identify the significantly different groups at ($P<0.05$) by one-way ANOVA test using SPSS software statistical program (SPSS for windows ver. 21.00, USA).

Results and Discussion

In this present study *A. platensis* was grown in five different media which contain variable concentration of sucrose as carbon source. OD values were determined depending on sucrose concentration changes (Figure 1). These results suggest that the *A. platensis* is adapting to the mixotrophic condition. At low sucrose concentrations, stationary phase was reached in later days (16^{th}) of incubation. At lower sucrose concentrations of less than 2.5 mM, the specific growth rate was lower, while the rising sucrose concentrations increased the specific growth rate (Figure 2). The highest specific growth rate (0.118 day^{-1}) was detected in 2.5 mM sucrose medium ($p<0.05$). Similarly, in a study the highest specific growth rate was detected in mixotrophic cultivation with 2.5 g/L glucose (Chojnacka, & Noworyta, 2004).

In other production media the specific growth rates were detected as 0.091 day^{-1} (with 0 mM sucrose), 0.102 day^{-1} (with 0.25 mM sucrose), and 0.046 day^{-1} (with 10 mM sucrose), (as the specific growth rate with 50 mM sucrose medium was lower, it was not shown in graph). In medium with high sucrose concentration (10 or 50 mM), the cell has mass growth and may not have gone into cell division. 2.5 fold decrease was detected in specific growth rate with 4 fold increasing sucrose concentration ($p<0.05$). In mixotrophic culture, autotrophic and heterotrophic metabolism were work together. The cyanobacteria cells were reached stationary phase rapidly and there were no significant changes in OD values. For this reason, specific growth rate of the medium with high sucrose concentration was detected lower than control condition (phototrophic cultivation).

In this present work, a higher growth rate was achieved in the mixotrophic medium than in the control condition because of low light intensity (1500 lux) and the presence of external carbon source. Even in phototrophic cultivation of *A. platensis* the optimal light intensity is 2500 lux, the light intensity in control condition (1500 lux or 20.25 µmol photon m^{-2} s^{-2}) was deficient. The light intensity in mixotrophic culture was sufficient as there was external carbon source. At high sucrose concentrations, substrate inhibition was also determined. The growth rate in the mixotrophic medium containing 10 mM sucrose was about 2 times lower than in the control condition ($p<0.05$). As a result of reaching rapidly to the specific growth rate, substrate inhibition was detected.

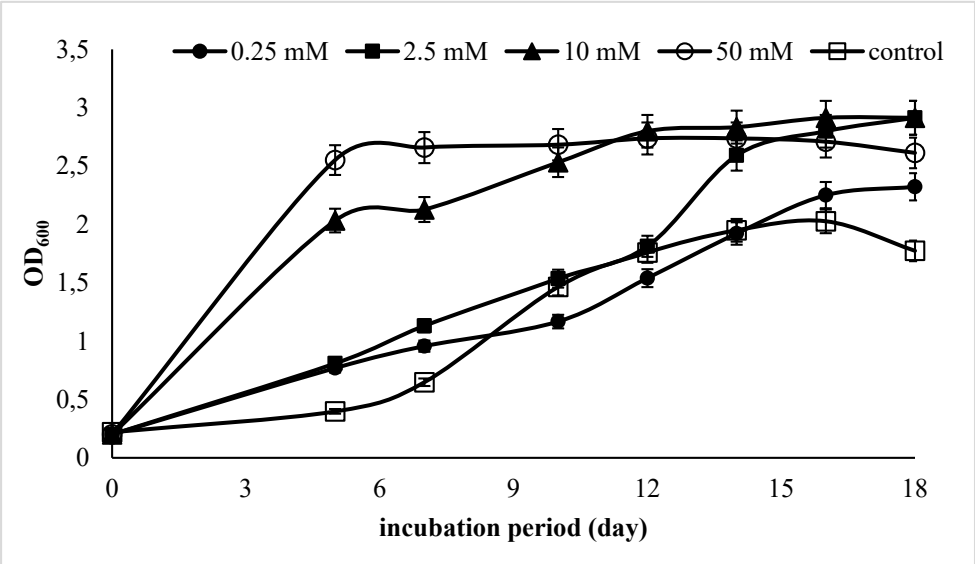

Figure 1. Variations of OD in *A. platensis* in different sucrose concentration medium depending on incubation period.

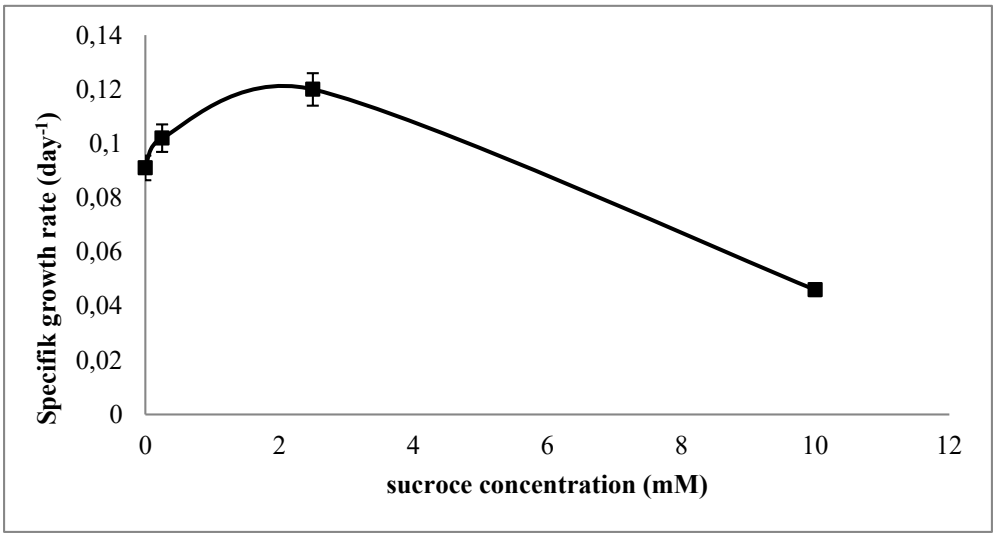

Figure 2. Specific growth rate values in varying sucrose concentrations media.

Additionally, the highest biomass productivity was detected as 1.33 g/L/day in the medium containing 2.5 mM sucrose. The biomass productivity of *Arthrospira* sp. varies from 0.06 to 4.3 g/L/day depending on the species (Mata et al., 2010). This result shows that *A. platensis* adapted to sucrose containing medium (2.5 mM) and reached to a high production rate. An increase was detected in biomass productivity with increasing sucrose concentration up to 2.5 mM. These results indicate that *A. platensis* cannot be adapted to high sucrose concentrations. However, the sucrose concentration up to 2.5 mM provided better growth than the control condition. When the medium supplemented with external carbon source, C availability can exceed cell necessities for growth and the rest carbon is directed towards lipid or carbohydrate synthesis (Lari et al., 2016). Generally specific growth rate of mixotrophic culture is the sum of phototrophic and heterotrophic metabolism because the external organic carbon promotes faster growth (Perez-Garcia et al., 2011). It can be said that, under controlled condition the specific growth rate is lower in the mixotrophic medium containing sucrose up to 2.5 mM than the controlled media ($p<0.05$).

Different kinds of simple sugars like glucose, fructose, galactose, mannose, lactose and sucrose support the mixotrophic and heterotrophic growth of cyanobacteria with species-specific differences in uptake and assimilation mechanisms (Neilson & Lewin, 1974; Shi et al., 1999; Sun et al., 2008). The study of the effect of different sugars and concentrations on the growth of *Arthrospira* sp. have shown that sucrose does not support growth in the dark but is effective in growing for certain species in the light conditions (Mühling et al., 2005). In this study, bleaching was detected in *A. platensis* cultures during adaptation to sucrose medium, and present study shows similarity in high sucrose concentrations (50 mM). Sucrose, trehalose and glucosyglycerol are osmoprotective compounds. The cyanobacterium *Synechocystis* sp. has active transport mechanism for glucosyglycerol and in salt-adapted cells is mainly achieved by *de novo* synthesis of the transport system (Mikkat et al., 1996; Mikkat et al., 1997). The studies support that trehalose and sucrose are taken up by the cells and possesses nearly the same as glucosylglycerol.

The inhibitory effect of the 50 mM sucrose concentration is also evident from the level of Chl-a and Chl-b content (Figure 3-4). The highest Chl-a content (301.173 ±14.8 mg/ g cell) was detected in the 2.5 mM sucrose containing medium, while the highest Chl-b content (42.62 ±1.9 µg/ mg cell) was detected under phototrophic cultivation. The chlorophyll amount did not differ significantly between control condition and mixotrophic cultivation (with 2.5 mM sucrose). In the study of Gim et al. (2016), the chlorophyll concentrations had no meaningful changes in mixotrophic (with 20 mM glucose) and phototrophic cultures.

In phototrophic culture the sole carbon source was CO_2 and the cyanobacteria needed chlorophyll to produce nutrient by using light and CO_2. On the contrary, in mixotrophic condition CO_2 was not the unique factor that supported biomass production. The mixotrophic condition is identified as "two-stage" mode (Zhan et al., 2017). The first stage is heterotrophy due to high content of initial organic carbon. When the organic carbon reduces to a certain level, phototrophic metabolism gets involved as first stage. Increasing amount of Chl-a in the late days of incubation under mixotrophic condition is associated with an increase in the amount of cells and phototrophic metabolism. And also the decreasing of available organic carbon load in the medium turns the mixotrophic metabolism to phototrophic metabolism.

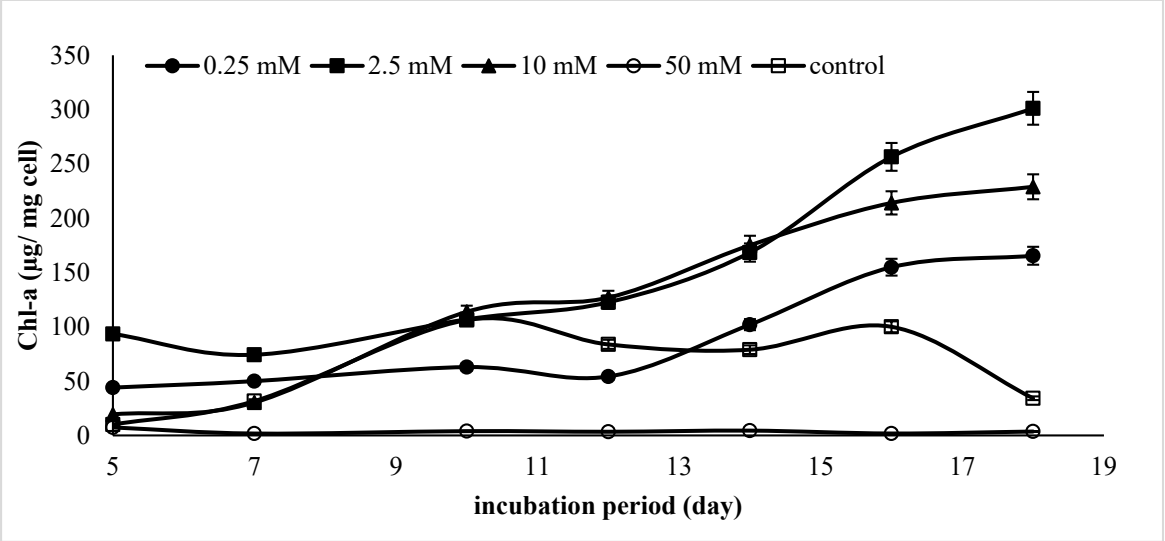

Figure 3. Chlorophyll-a content of *A. platensis* in different sucrose concentration medium depending on incubation period.

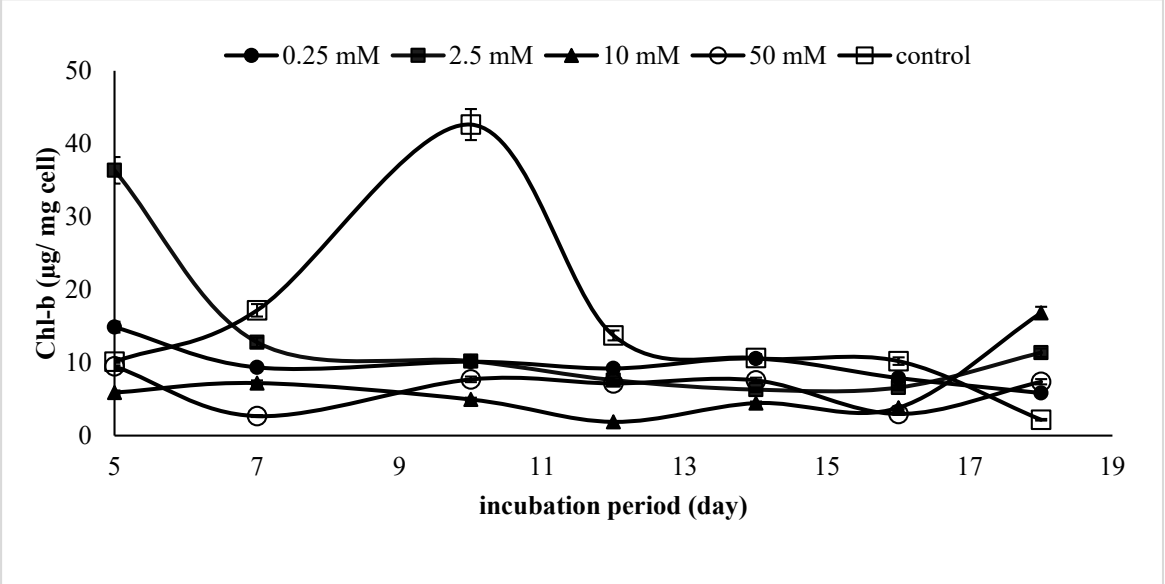

Figure 4. Chlorophyll-b content of *A. platensis* in different sucrose concentration medium depending on incubation period

There was approximately 1.5 unit overall increase in pH values during the incubation period (Figure 5). The determined pH value increase may be due to organic bases released into the medium during the production process. Only in the presence of 50 mM sucrose, there was a decrease with a fluctuation in the pH value. In this medium, excess carbon source may inhibit the cellular metabolism, hence the disaccharide is not rapidly breakdown.

Insignificant lipid production was detected at minimum (control and 0.25 mM) and maximum (50 mM) sucrose concentrations ($p>0.05$) (Figure 6). While lipid production varied with increasing sucrose concentration, the highest lipid content (3.68 ±0.17 mg/ g cell) was determined on the 16th day of incubation in medium containing 2.5 mM sucrose ($p<0.05$). In control condition and the medium containing 0.25 mM sucrose, the maximum amount of lipid was detected in the first days of incubation. In medium with high sucrose

concentration, lipid production was increased late in the incubation days because the cells provided later adaptation. When the sucrose concentration was higher than 2.5 mM, substrate inhibition was observed. In a study two different microalgae were grown in mixotrophic culture including glucose (Cheirsilp & Torpee, 2012). The lipid content of both strains decreased sharply when the initial glucose concentration increased from 0 to 4 g/L. At above 4 g/L of initial glucose concentration, the lipid content did not change significantly. Similarly, in the study of Lin and Wu (2015), lipid production of *Chlorella* sp. increased when the initial sucrose concentration increased to 0.5 g/L. When the initial sucrose concentration was higher than 0.5 g/L, the lipid production decreased.

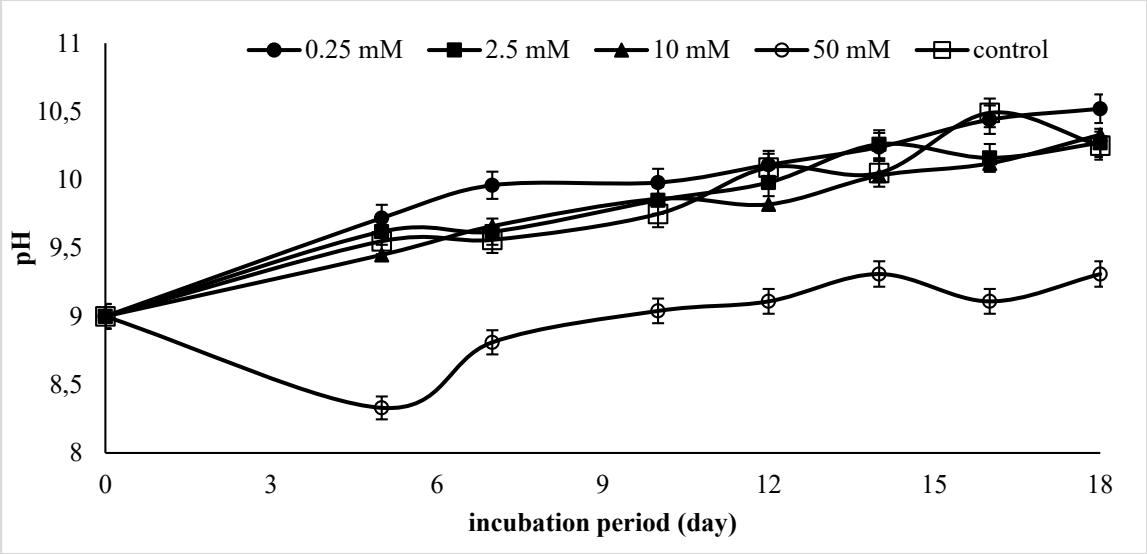

Figure 5. Variations of pH of *A. platensis* in different sucrose concentration medium depending on incubation period.

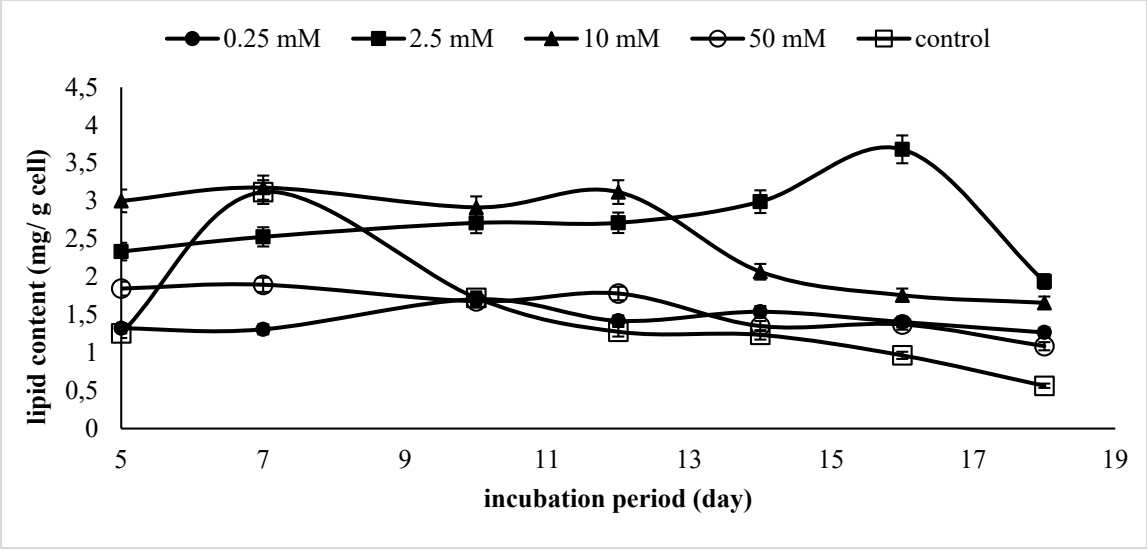

Figure 6. Lipid content of *A. platensis* in different sucrose concentration medium depending on incubation period.

Table 1. Comparison of control condition (phototrophic) and mixotrophic culture (containing 2.5 mM sucrose) that has been determined the best results.

Parameters	mixotrophic culture	control (phototrophic culture)
Specific growth rate (day^{-1})	0.118	0.091
Biomass productivity (g/L/day)	1.33	0.153
Lipid content (mg/ mg cell)	3.68 ±0.17	3.118 ±0.14
Chl-a content (µg/ mg cell)	301.173 ±14.8	106.303 ±4.9
Chl-b content (µg/ mg cell)	36.362 ±1.7	42.62 ±1.9

Values are mean ± S.D., N = 3; ($p < 0.05$).

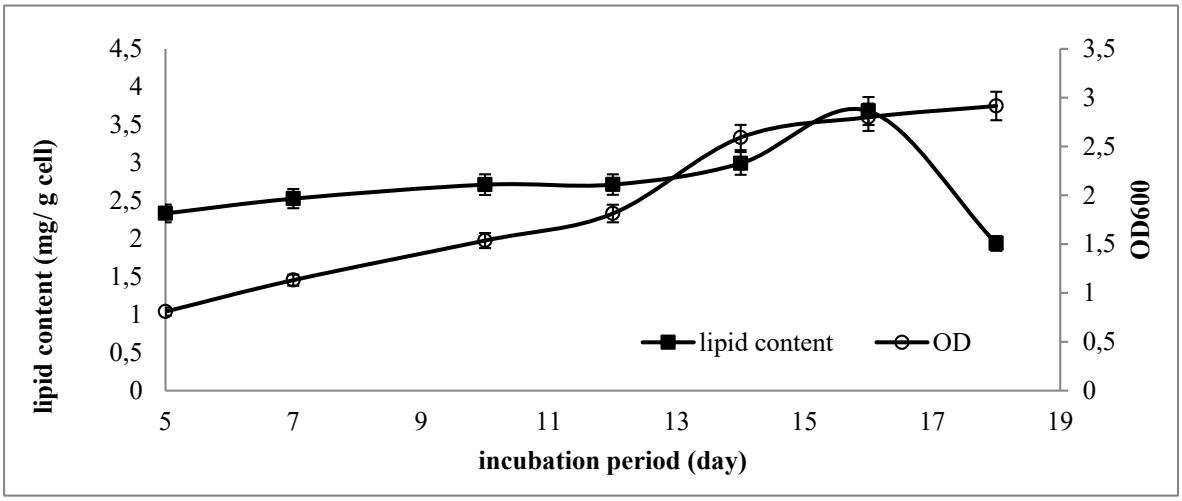

Figure 7. Lipid content and OD of *A. platensis* in 2.5 mM sucrose medium depending on incubation period.

According to the obtained results, the highest specific growth rate, biomass productivity, Chl-a and lipid content was detected in mixotrophic culture that contains 2.5 mM sucrose (Table 1). These results showed that sucrose including medium supports biomass and chlorophyll production and lipid accumulation of *A. platensis*.

In the highest lipid production condition, biomass and lipid content according to the incubation period is shown in Figure 7. According to figure it was determined that the maximum amount of lipid was obtained at the stationary phase. This can be interpreted as *A. platensis* culture grown with adaptation of sucrose has increased lipid production by entering the stress due to reduced external carbon source in the medium. In this medium on the 16th day of incubation, external and intracellular total carbohydrate concentration was detected as 28.29 ppm and 130.26 µg/g cell, respectively.

The thermal stability of the produced cell was investigated by TGA and DSC. The thermal stability of produced cell could show difference according to production medium. The produced cyanobacterium cells in different media have almost same degradation profile. In the first step, 2.19% of weight loss for phototrophic production and 4.13% of weight loss for mixotrophic production were recorded. The maximum degradation was determined in the second step for two of them. The weight loss was 52.94% and 54.74% for phototrophic and mixotrophic production, respectively. The most important difference was the cyanobacterium cell that produced in phototrophic culture has showed more rapid weight loss than produced in mixotrophic culture. That means the cyanobacterium cell produced in mixotrophic culture has higher thermal stability.

Also the functional groups of the produced cyanobacterium were investigated by FTIR. In FT-IR spectra of cyanobacterium cell produced phototrophic and mixotrophic cultures there were same peaks such as 2990-2924 cm^{-1} showed CH_3 asymmetric stretching which was associated with lipid, carbohydrate or protein structure, 1650 cm^{-1} relevant with C=O stretching on protein structure and the peak belongs to N-H bending and C-N stretching next to 1542 cm^{-1}, 1240 cm^{-1} related to asymmetric stretching of hydrocarbon chain and

phospholipid structures. The peak at 2856 cm^{-1} was formed as a result of CH_2 symmetric resonance in lipid and carbohydrate structure which was detected only cyanobacterium cell produced in mixotrophic culture FTIR spectrum. This result supports that more lipid production was produced in the mixotrophic culture.

Conclusions

In conclusion the present study suggests a new carbon source for mixotrophic culture of *A. platensis* using sucrose. *A. platensis* has adapted to the medium that contains low sucrose concentrations. Significant decrease was detected in specific growth rate with increasing sucrose concentration ($p<0.05$). In the mixotrophic cultivation of *A. platensis* two different metabolic pathways were active. Due to there was sufficient external carbon source in the first days of incubation, heterotrophic metabolism was used more actively. When the organic carbon source sucrose reached a critical concentration chlorophyll content started to increase. That means heterotrophic and phototrophic metabolism worked correlated. The produced cells in mixotrophic culture (with 2.5 mM sucrose) has higher thermal stability depending on TGA. Additionally, this study showed that sucrose containing medium supports lipid production. And this result is supported by the FTIR spectrum. In this present study, it can be said that *A. platensis* can use sucrose as a carbon source. This result is an indication that various wastes containing sucrose such as molasses, sugar cane bagasse can also be used as a carbon source in the production medium. Thus, valuable products such as biomass, protein, and lipid can be produced more economically and hence used economically for in various industrial areas such as food, fisheries, and pharmaceuticals. The produced *A. platensis* biomass would be evaluated as protein and lipid source in aquaculture diets.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: There is no need ethics committee approval.

Financial disclosure: The authors declare that for this article there are no financial support.

Acknowledgments: We would like to thank Assoc. Dr. Leyla Uslu for her supply of cyanobacteria.

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