

Lipid production in *Dunaliella bardawil* under autotrophic, heterotrophic and mixotrophic conditions

Zohreh Zare Chavoshi¹, Mansour Shariati^{1*} 

¹ University of Isfahan
(Department of Biology, Hezar Jarib St, Isfahan, Iran).

*Corresponding author: mansour_shariati@yahoo.com
mansour@sci.ui.ac.ir

ABSTRACT

Many microalgae are rich in lipid. Due to their low growth rates in the autotrophic culture, the best alternative is to cultivate cells under different conditions such as heterotrophic or mixotrophic, which results in the highest yield of biomass and lipid in the shortest duration. In this study, *Dunaliella bardawil* (UTEX 2538) green microalgae was cultivated under different culture conditions, autotrophic, heterotrophic and mixotrophic, and effects on cell concentration, lipid production and reactive oxygen species (ROS), total chlorophyll and beta-carotene concentrations were investigated. Due to very low cellular growth, this alga is not recommended for heterotrophic culture. In terms of mixotrophic conditions containing different concentrations of glucose or acetate, the highest cell growth and lipid production in 60mM glucose was similar to the control (autotrophic condition), while the concentrations of chlorophyll and beta-carotene decreased. However, at all concentrations of acetate, a slight increase in cell growth was achieved, while the lipid content decreased. Additionally, the concentrations of chlorophyll and beta-carotene increased. A positive correlation was observed between beta-carotene biosynthesis and lipid production, as well as levels of reactive species of oxygen and lipid production in the presence of glucose and acetate. This study showed that for *D. bardawil* the mixotrophic culture with 60mM glucose was the most suitable type of culture for increasing lipid content and cell growth rates in less time (one week).

Descriptors: beta-carotene, cell growth, chlorophyll, culture condition, reactive oxygen species.

INTRODUCTION

Lipids are vital for the growth and survival of all organisms. They are important structural compounds of cell membranes and play an important role as energy and carbon reservoirs (De Swaaf et al., 2003). The accumulation of lipids in microalgae depends on several factors (Takagi and Karseno, 2006), including growth conditions (Schenk et al., 2009), whether inorganic or organic carbon sources are used (De Swaaf et al., 2003), and specific strains of microalgae (Chisti, 2007). Most microalgae growth under autotrophic conditions, but subject to low biomass production, low lipid content and a long period of cultivation to reach exponential growth (Martinez and Orus, 1991). Therefore, heterotrophic and mixotrophic cultures have been proposed as alternatives for mass biomass production and cellular lipid accumulation in microalgae (Yu et al., 2009).

The heterotrophic culture involves the use of organic compounds such as sugars and organic acids as the only

carbon and energy sources in the absence of light (Chojnacka et al., 2004). Some studies have shown that such approach may increase the production of biomass and lipids in microalgae (Miao and Wu, 2006; Boyle and Morgan, 2009). In mixotrophic cultures, organic and inorganic carbon sources are used simultaneously in the presence of light (Chojnacka et al., 2004), and photosynthesis and respiration occur simultaneously (Lee, 2004; Sun et al., 2008).

In the pathway for lipids biosynthesis (Ren et al., 2009), acetyl coenzyme A (acetyl-CoA) acts as a precursor. Cytosolic storage pathways for acetyl-CoA are dependent on organic carbon sources used for growth, such as glucose and acetate. The main route of carbon from glucose to cytosolic acetyl-CoA includes glycolysis, the transfer of pyruvate to mitochondria, the conversion of pyruvate to citrate, transfer of citrate to cytosol, and the conversion of citrate by ATP-citrate lyase to acetyl-CoA. The conversion of acetate to acetyl-CoA involves a one-step enzyme reaction catalyzed by acetyl-CoA synthase (Ratledge and Evans, 1989).

Dunaliella is a single-cell green alga belonging to the class Chlorophyceae (Capa-Robles et al., 2009). Due to the lack of cell wall in *Dunaliella*, the extraction of

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produced materials is much cheaper than other algae and plants (Hosseini Tafreshi and Shariati, 2006). *Dunaliella* species are able to survive in a wide range of salinity (Ben-Amotz, 2004) due to their ability to collect large amount of glycerol (Ben-Amotz et al., 1982). Some *Dunaliella* species such as *D. bardawil* (= *D. salina*; Borowitzka, 2016) can produce high amounts of beta-carotene (Vanitha et al., 2007) in response to some stress conditions such as intense light, high salinity, and low nutrient concentration (Hosseini Tafreshi and Shariati, 2006). *Dunaliella* species are able to store lipids (Gouveia and Oliveira, 2009) as well as glycerol, which is used as a lipid storage in microalgae in biosynthesis pathway of triacylglycerides (Sharma et al., 2012). Therefore, optimizing the growth conditions are essential for high lipid production (Borowitzka, 1999).

Hence, in this study, the effects of different nutritional conditions (autotrophic, heterotrophic and mixotrophic cultures), as well as organic carbon inputs (glucose and acetate) on the cell growth and production of *Dunaliella bardawil* (= *D. salina*) were investigated. We aimed to identify culture conditions that can lead to the highest levels of lipid production in *D. bardawil* (= *D. salina*) in the shortest time interval, without reducing cell growth rates.

MATERIAL AND METHODS

MICROALGAL STRAIN AND CULTURE MEDIUM

Dunaliella bardawil (= *D. salina*) was obtained from the University of Texas Culture Collection (UTEX 2538). The original cultures were maintained in modified (Shariati and Lilley, 1994) Johnson's solution (Johnson et al., 1968), composed of KNO₃ 5mM, CaCl₂ 0.2mM, MgSO₄·7H₂O 5mM, KH₂PO₄ 0.2mM, CuCl₂ 1μM, ZnCl₂ 1μM, MnCl₂ 7μM, (NH₄)₆Mo₇O₂₄·4H₂O 1μM, CoCl₂ 1μM, FeCl₃·6H₂O 4μM, Na₂EDTA 10μM, NaCl 1.5 M. The pH of the culture medium was adjusted in the range of 7-7.5. The culture medium was sterilized in an autoclave for 15 min at 121 °C. The cells were cultured in 100 mL of the culture medium, and were inoculated with 10% (v/v) cells. The cultures were kept at 27 °C, 16/8h light/dark regime under continued shaking at 110 rpm (INFORS shaker AG CH - 4103, Bottmingen).

CULTURE METHODS

For the control (autotrophic condition) culture, 25mM of NaHCO₃ were added to the culture medium as inorganic carbon source, at a fluorescent light intensity of 100μmol photons m⁻² s⁻¹ (SQPR Hansatech, UK) and a 16/8 h

light/dark photoperiod for 14 days. For the heterotrophic culture, the organic carbon sources glucose (at zero, 20, 40, 60 and 80mM) and acetate (at zero, 50, 100 and 150mM) were used. The algal cultures were placed under dark condition and wrapped in aluminum foil, for 14 days. The mixotrophic culture was kept under the same light and inorganic carbon conditions as in the autotrophic culture, and organic carbon was added as glucose (at zero, 20, 40, 60 and 80mM) and acetate (at zero, 50, 100 and 150mM). After autoclaving the culture medium, glucose and acetate solutions were filtered separately using 0.22μm pore size filter membrane and added to the culture whenever needed.

ANALYTICAL METHODS

Sampling for cell counting and measuring chlorophyll and beta-carotene concentrations was carried out at the onset of the experiments and after 4, 7, 10 and 14 days. The total lipid content was measured at the beginning of the experiment and after 7 and 14 days.

To measure the cell concentration, 200μl of the algal suspension were removed and 10μl of formaldehyde solution was added; counts were performed with a hemocytometer under a regular microscope (Olympus CH 3 ORF 200). The final result was calculated as cells per mL (Schoen, 1988).

Chlorophyll and beta-carotene concentrations were estimated in 1mL of algal culture, after centrifugation at 13000 rpm for 6 min (Eppendorf AG 22331 Hamburg). The supernatant was removed and 1mL of 80% acetone was added to the pellet. After centrifugation at 13000 rpm for 4 min, the absorption of the supernatant was read using a spectrophotometer (Shimadzu UV-160A) at 412, 431, 460 and 480nm, and chlorophyll a (Chla), chlorophyll b (Chlb) and beta-carotene concentrations were estimated with the following equations (Eijkelhoff and Dekker, 1997):

$$\text{Chl}_a = -1.709A_{412} + 11.970A_{431} - 2.998A_{460} - 5.708A_{480} \quad (1)$$

$$\text{Chl}_b = -0.171A_{412} - 0.230A_{431} + 11.871A_{460} - 13.248A_{480} \quad (2)$$

$$\text{Total chlorophyll} = \text{Chl}_a + \text{Chl}_b \quad (3)$$

$$\text{Beta - Carotene} = -0.430A_{412} + 0.251A_{431} - 13.216A_{480} \quad (4)$$

Total lipids were analyzed after centrifugation at 2000 rpm for 15 minutes (IEC HN-S centrifuge). The supernatant was removed carefully, the pellet was weighed as fresh weight and kept at -20 °C. The total lipid was extracted from the fresh cells according to Bligh and Dyer (1959) with a mixture of chloroform:methanol:NaCl 1%

(2:1:2 v/v). The pellet was heated in a microwave to break down the cell membranes. Methanol was added, the pellet vortexed for 5 minutes, then chloroform and NaCl 1% were added. The mixture was centrifuged at 2000 rpm for 10 minutes to obtain a clear supernatant. The supernatant was removed and the bottom solution was dried using N₂ bubbling, which included lipid and chloroform. After complete drying, the remaining material was weighed to obtain the amount of total lipid per fresh weight biomass.

The method of Mahalingam et al. (2006) was applied to measure the total ROS (reactive oxygen species) levels. The algal suspension was harvested, cells were centrifuged (IEC HN-S centrifuge) at 2000 rpm for 15 minutes, and then the supernatant was removed. The precipitate was mixed with 10mM Tris-HCl buffer (pH 7.2). The suspensions were homogenized by the ultrasonic method for 10 minutes. Finally, the obtained homogenates were centrifuged at 14000 rcf for 20 minutes at 4° C. The supernatant (900µL) was mixed with 1mM 2', 7'-dichlorofluorescein diacetate (DCFDA) solution in dimethyl sulfoxide (DMSO) and vortexed for 30 seconds. The samples were placed in dark for 15 minutes and fluorescence was measured with a spectrofluorometer (F90 fluorescence spectrophotometer) at 480nm. The total protein concentration of samples was measured using the Biuret method (Gornall et al., 1949). Data were expressed as relative fluorescence units (RFU) per g mL⁻¹ of protein.

STATISTICAL ANALYSIS

All experiments were performed at least in three replicates. The data were expressed as mean ± SD (standard deviation). The normality tests (Descriptive Statistics/Frequencies) were performed before ANOVA. Differences between treatments were determined at $p < 0.05$ using factorial analysis of variance (one-way ANOVA) with a Tukey multiple comparisons post hoc test. Cell growth, chlorophyll and beta-carotene concentration were compared between treatments using repeated-measures ANOVA. Data of total chlorophyll concentration under treatment of glucose, were not normal, therefore, differences between treatments were determined at $p < 0.05$ using Kruskal-Wallis. All statistics were carried out using the SPSS software (version 23 for Windows; SPSS Inc., Chicago, IL, USA).

RESULTS

THE EFFECTS OF GLUCOSE AND ACETATE AS TWO DIFFERENT SOURCES OF ORGANIC CARBON

In this study, optimization of two organic carbon sources namely glucose and acetate was studied under

the autotrophic (control, no glucose), mixotrophic, and heterotrophic conditions. In the presence of glucose (Figure 1A), cell concentration in all treatments was similar until day 4 of experiment. After 1 week, a high increase was observed at glucose concentrations of 40, 60 and 80mM, which corresponded to 45, 33 and 33×10⁶ cells per mL, respectively. By increasing glucose concentration above 60mM, a significant decrease (26%) in cell number was observed. Until day 4, no difference was found in chlorophyll and beta-carotene concentrations between the control and the various glucose treatments (Figures 1B and 2A). After two weeks, chlorophyll decreased from 20 (control) to 5µg mL⁻¹ (Figure 1B), and beta-carotene went from 8 (control) to 3µg mL⁻¹ (Figure 2A). An increase was found in lipid production after one week under glucose treatments of 20, 40 and 60mM, but lipid production returned to initial levels after two weeks, in all glucose concentrations (Figure 2B). After two weeks, a decrease was observed in lipid production (from 0.22 to 0.1) with all glucose concentrations.

By using acetate in the mixotrophic culture, a slight increase in cell concentration was observed (Figure 3A) compared to the autotrophic condition (control, no acetate). In all concentrations of acetate (50, 100 and 150mM), total chlorophyll (Figure 3B) and beta-carotene

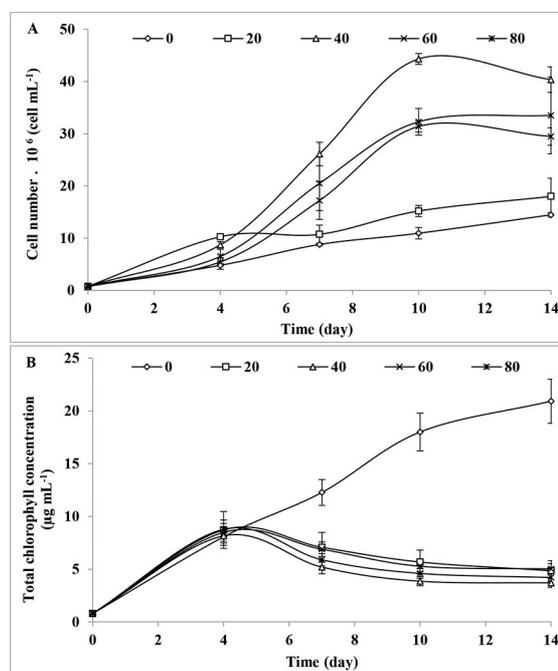


Figure 1. Effects of different concentrations of glucose (mM) on A. Cell number (cell mL⁻¹), and B. Total chlorophyll concentration (µg mL⁻¹) of *Dunaliella bardawil* (= *D. salina*) in mixotrophic culture in comparison with autotrophic condition (control = zero concentration). The data are expressed as mean ± standard deviation of three replicates.

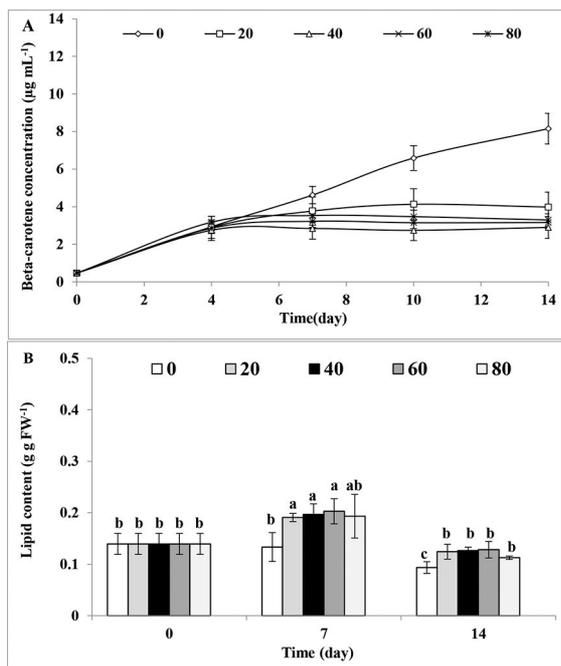


Figure 2. Effects of different concentrations of glucose (mM) on A. Beta-carotene concentration ($\mu\text{g mL}^{-1}$), and B. Lipid production (g g FW^{-1}) of *Dunaliella bardawil* (= *D. salina*) in mixotrophic culture in comparison with autotrophic condition (control = zero concentration). The data are expressed as mean \pm standard deviation of three replicates. Different letters in b indicate significant difference at $p < 0.05$ according to Tukey test.

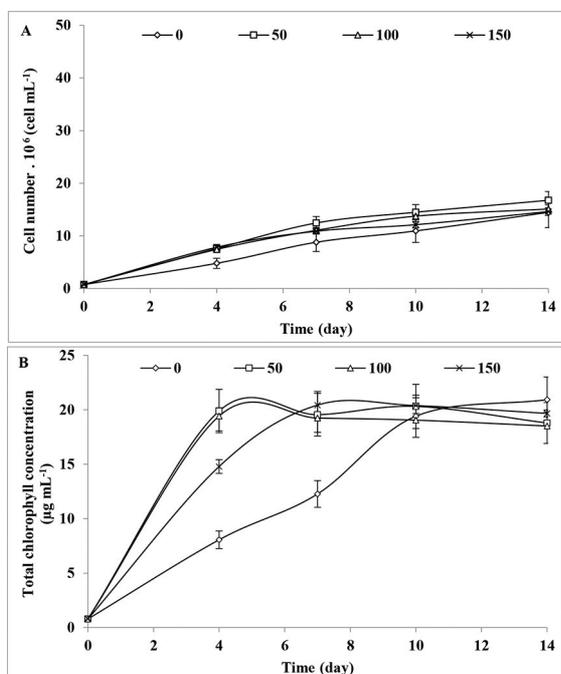


Figure 3. Effects of different concentrations of acetate (mM) on A. Cell number (cell mL^{-1}), and B. Total chlorophyll concentration ($\mu\text{g mL}^{-1}$) of *Dunaliella bardawil* (= *D. salina*) in mixotrophic culture in comparison with autotrophic condition (control = zero concentration). The data are expressed as mean \pm standard deviation of three replicates.

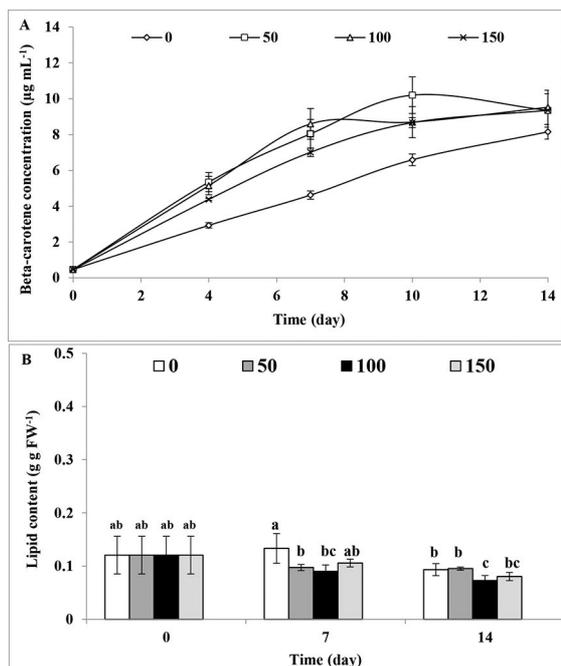


Figure 4. Effects of different concentrations of acetate (mM) on A. Beta-carotene concentration ($\mu\text{g mL}^{-1}$), and B. Lipid production (g g FW^{-1}) of *Dunaliella bardawil* (= *D. salina*) in mixotrophic culture in comparison with autotrophic condition (control = zero concentration). The data are expressed as mean \pm standard deviation of three replicates. Different letters in B indicate significant difference at $p < 0.05$ according to Tukey test.

concentrations (Figure 4A) showed a significant increase ($p < 0.05$). During 4 days, the highest increase in total chlorophyll was obtained at 20 $\mu\text{g mL}^{-1}$ at concentrations of 50 and 100mM, indicating a three- time increase compared to the control. After 1 week, the concentration of chlorophyll at 150mM of acetate reached about 20 $\mu\text{g mL}^{-1}$ (Figure 3B), similar to other concentrations of acetate in the medium. Finally, after 2 weeks, the total chlorophyll concentration was similar at all concentrations of acetate and control (autotrophic condition), and it was about 20 $\mu\text{g mL}^{-1}$. As shown in Figure 4A, beta-carotene concentration at all concentration of acetate increased about 7.8 $\mu\text{g mL}^{-1}$ (day 7), and 9.2 $\mu\text{g mL}^{-1}$ (day 10) compared to the control (corresponding values of 4.6 and 6.5, respectively). By increasing the concentrations above than 100mM, the trend of increase in total concentrations of chlorophyll decreased from 20 to 13 $\mu\text{g mL}^{-1}$ (within 4 days), and for beta-carotene, it decreased (within 7 days) from 8 to 7 $\mu\text{g mL}^{-1}$. As shown in Figure 4B, using acetate in the culture medium resulted in a decrease in lipid production about 21% in all different concentrations of acetate over two weeks ($p < 0.05$). After 4 days, the highest increase in total chlorophyll (20 $\mu\text{g mL}^{-1}$) was obtained at acetate

concentrations of 50 and 100mM, indicating a three-time increase compared to the control (Figure 3B). The same chlorophyll concentration was attained only after 1 week at 150mM of acetate (Figure 3B). After 2 weeks, the total chlorophyll concentration was similar at all concentrations of acetate and control (autotrophic condition), leveling at 20 $\mu\text{g mL}^{-1}$. Beta-carotene concentration at all concentrations of acetate increased compared to the control (Figure 4A). By increasing the acetate concentration above 100mM, beta-carotene decreased from 8 to 7 $\mu\text{g mL}^{-1}$ within 7 days. Using acetate in the culture medium resulted in a 21% decrease in lipid production over two weeks, regardless of acetate concentration.

Under heterotrophic condition (no light) and various concentrations of glucose and acetate, cell concentration, chlorophyll content and beta-carotene concentration were always lower than the control, represented by the autotrophic condition. Heterotrophic and autotrophic concentration maxima for the above-mentioned parameters were, respectively, $2.5 \times 10^6 \text{ cell mL}^{-1}$ and $13 \times 10^6 \text{ cell mL}^{-1}$ (cell concentration), $1\mu\text{g mL}^{-1}$ and $20\mu\text{g mL}^{-1}$ (chlorophyll), and $0.5\mu\text{g mL}^{-1}$ and $7\mu\text{g mL}^{-1}$ (beta-carotene). In various acetate and glucose concentrations, all the measured parameters were close to zero (data not shown). Therefore, lipid content was not estimated for the heterotrophic condition.

As shown in Figure 5A, after 4 days, the production of ROS had a slight increase of about 28% at a glucose concentration of 80mM. After 1 week, the ROS level decreased to 100 RFU/g mL^{-1} protein at all concentrations of glucose and acetate. No significant changes were observed in the total ROS levels 4 days after addition of 150mM acetate (Figure 5B).

DISCUSSION

Microalgae may store a large part of their dry weight as lipids (Chisti, 2008), and culture optimization is needed to enhance lipid production for industrial and nutritional applications. Considering cellular self-shading and photo-inhibition in autotrophic cultures (Kim et al., 2013) and the consequent reduction of biomass production, heterotrophic and mixotrophic cultures could be envisaged as alternatives to increase lipid content (Liang et al., 2009; Bumbak et al., 2011). However, the present results showed that cell concentration of *D. bardawil* (= *D. salina*) is very low in heterotrophic conditions with various concentrations of glucose and acetate. This is

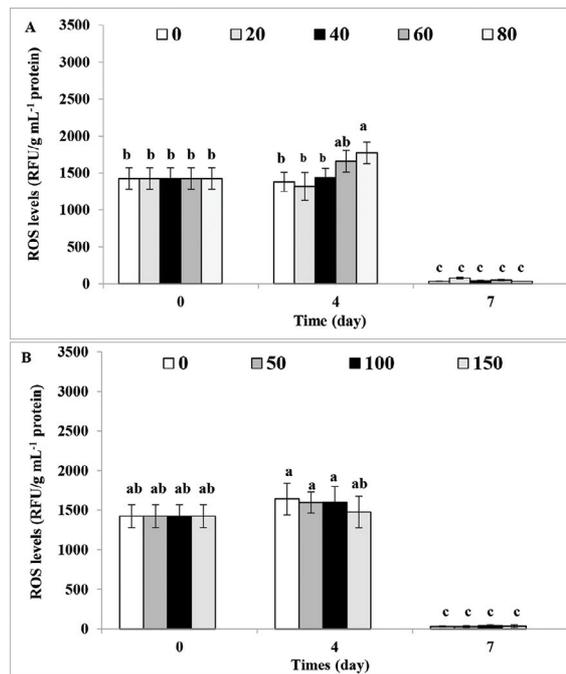


Figure 5. The intracellular ROS levels (RFU/g mL^{-1} protein) in the presence different concentrations of A. Glucose (mM), and B. Acetate (mM) in *Dunaliella bardawil* (= *D. salina*) in comparison with autotrophic condition (control = zero concentration). The data are expressed as mean \pm standard deviation of three replicates. Different letters indicate significant difference at $p < 0.05$ according to Tukey test.

not surprising, since *Dunaliella* is an obligated autotroph (Subba Rao, 2009), and the growth rate of *Dunaliella* species has been previously reported as very low in heterotrophic conditions (Hard and Gilmore, 1996).

High growth rates were observed at different concentrations of glucose (mixotrophic condition), compared to the control (autotrophic condition). In mixotrophic conditions, due to the presence of glucose as an organic carbon source, cell growth increased and length of growth period decreased, as cell division was not entirely dependent on photosynthesis (Cheirsilp and Torpee, 2012). The presence of light and organic carbon, as simultaneous sources of energy in mixotrophic conditions, allows both autotrophic and heterotrophic processes to take place (Facundo et al., 1993), leading to an increase in the growth rate. Organic carbon sources are expected to reduce CO_2 fixation through photosynthesis. In such scenario, while cell division increases, the metabolic cost of respiration may exceed the gain from photosynthesis (Liu et al., 2009; Rym et al., 2010). Photosynthetic microorganisms can use glucose in their metabolism as the final production of photosynthesis. Glucose has more energy per mole than other substrates (Boyle and Morgan, 2009) and seems to stimulate cell

growth (Garcia et al., 2005). Higher growth and respiration rates were reported with the use of glucose compared to other substrates, such as sugars and organic acids (Griffiths et al., 1960). High concentrations of glucose (> 60mM) reduce cell division, which may be explained by substrate inhibition of some enzymes (Garcia et al., 2005; Reed et al., 2010).

The lower concentrations of chlorophyll and beta-carotene in the presence of glucose in mixotrophic conditions are likely to be related to an increase in the heterotrophic metabolism of glucose (Shihira-Ishikawa and Hase, 1964), and to the destruction of chlorophyll *a* in response to a decrease in the number of thylakoid membranes in chloroplast (Stadnichuk et al., 1998; Anila et al., 2013). The increase in ROS levels in the presence of glucose seems to cause the chlorophyll degradation (Cuello and Lahora, 1993). Increase in the synthesis of chlorophyll and beta-carotene in *D. bardawil* (= *D. salina*) can be due to the presence of acetate, as precursor of acetyl CoA (Murphy and Walker 1982). Acetyl CoA is a precursor of beta-carotene synthesis pathway (Sasso et al., 2012) in several organisms (Lichtenthaler, 1999), including *Dunaliella* (Ben-Amotz and Shaish, 1992). It also acts in the biosynthesis of phytol, a lateral chain of chlorophylls (Paniagua-Michel et al., 2012). Seemingly, acetate contributes in the beta-carotene and chlorophyll synthesis by producing acetyl CoA.

In the presence of glucose in mixotrophic conditions after 1 week, the lipid levels were about 1.5 times higher than in the autotrophic condition. Considering that acetyl-CoA is also a precursor in lipid biosynthesis pathways (Ren et al., 2009), it seems that glucose as an organic carbon source influences lipid production by providing additional energy and products, such as Acetyl-CoA and NADPH, which are essential for lipid accumulation in microorganisms (Ren et al., 2009). However, increase of lipid levels in the presence of glucose was associated with a decrease in beta-carotene concentration, lipid content decreased along with an enhancement in beta-carotene concentration in the presence of acetate. As lipids and beta-carotene have the same precursor (Paniagua-Michel et al., 2009), different conditions may influence carbon partitioning toward beta-carotene or lipid production (Juneja et al., 2013). Apparently, decrease in the beta-carotene concentration under the glucose treatment and increase in the beta-carotene concentration in the acetate treatment led to an increase and decrease in lipid content, respectively (Bonnefond et al., 2017).

Increase in the ROS production in response to high glucose concentration, and lack of changes in the presence of acetate, can also be attributed to the low and high beta-carotene concentration, respectively. It seems that in the presence of glucose, low concentrations of beta-carotene are not able to prevent ROS production (Jiménez and Pick, 1993).

CONCLUSION

The results of the present study on *D. bardawil* (= *D. salina*) cultivation showed that the mixotrophic culture containing glucose is the most suitable alternative for high cell growth and lipid production. The mixotrophic culture containing acetate was also found as a better alternative to high production of beta-carotene in a short time interval (about one week). As an obligate autotroph, *Dunaliella* has a very low cell division in the heterotrophic treatment, therefore lipid production is not recommended in such condition.

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