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Effects of Sodium Nitrate and Mixotrophic Culture on Biomass and Lipid Production in Hypersaline Microalgae *Dunaliella Viridis* Teod

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ABSTRACT

To access the potential application of Dunaliella viridis Teod. *for biofuel production, the effects of culture media composition on biomass and lipid content of this microalgae were investigated. Measured at the 20 th day, sodium nitrate at 5.0 mM augmented biomass production by 26.5 percent compared to control (1 mM sodium nitrate). Total lipids expressed as µg mL*$^{-1}$ *of culture also increased with increase in nitrate concentration up to 5.0 mM sodium nitrate, whereas when expressed on the per cell basis, total lipids stayed relatively constant at most of the tested nitrate concentrations except at 0.5 mM which was 31.4 percent higher compared to 1.0 mM nitrate. At 5.0 mM sodium nitrate, by using 20 g L*$^{-1}$ *of glucose in mixotrophic culture of D. viridis, cell number augmented by 36.4 percent compared to the cultures with no added glucose. Llipid content per cell and per mL of culture was increased by 71.4 and 135.1 percent, respectively. Among plant hormones,* 10^{-9} *M indole-3- acetic acid (IAA) plus* 10^{-8} *M trans-zeatin riboside led to 22.8 percent higher biomass relative to control (without hormone and at 1.0 mM sodium nitrate). It is concluded that altering the growth conditions of D. viridis can lead to higher cell densities and higher lipids content which can be exploited for biofuel production.*

Key words: *Dunaliella viridis*, mixotrophic culture, biofuel, biomass, lipid content, hormones

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INTRODUCTION

Microalgae are one of the sources of natural oils, polysaccharides, proteins, carotenoid pigments, vitamins and micronutrients (Michalak et al. 2014). They have many applications in the food industries (El Baky et al. 2013) and also have been used in biofuel production (Hu et al. 2008; Sheehan et al. 1998).

In the past decade, bioenergy production from microalgae has been the subject of many studies. A few microalgae species including some *Chlorella*, *Dunaliella*, *Nannochloris*, *Neochloris* and *Botryococcus* have the capacity for accumulating large quantities of lipids in their cells under favorable conditions (Li et al. 2008). Biofuel production from microalgae is dependent on the rate of microalgae biomass production and their lipid content (Chen et al. 2011). Some systems, like photo-bioreactors and open ponds are of particular importance for biofuel production (Sato et al. 2006).

From different stand point, such as depletion of resources and climate change, fossil fuels have started to show their limitations as an energy source. Therefore in near future, renewable energies need to become a dominant energy source, displacing the fossil fuels (Deng et al. 2011). Due to various reasons microalgae seems to be a promising source for biofuel production (Nigam et al. 2011; Brennan and Owende 2010). Oil productivity compared to oilseed crops, less need for fresh water than terrestrial crops, rapid growth rate, bio-fixation of CO_2, no need for herbicides or pesticides and no strain on agronomical crops and land use are among many factor that make microalgae suitable for biofuel production (Brennan and Owende, 2010). Manipulation of microalgae growth conditions for production of biofuel is a rapidly expanding area of research. Various studies have been carried out on lipid induction techniques in microalgae. Lipid induction may be performance by limitation in nutrient elements such as nitrogen, phosphorus, iron, potassium, sulfur and magnesium (Deng et al. 2011). Other environmental factors such as temperature stress, light intensity and salinity also can induce lipid production in some microalgae (Sharma et al. 2012). These factors also affect algal biomass which is an import factor in biofuel production (Tang et al. 2010; Yeesang and Cheirsilp 2011).

The genus *Dunaliella* is widely distributed in the world and its species live in an extremely wide range of habitats. For example, *D. lateralis* live in fresh waters while *D. salina* mainly exists in hypersaline environments (Borowitzka et al. 2007). *D. salina* and *D. viridis* are the predominant phytoplankton species in lakes with intermediate to high salinity levels (Davis 1990). To reduce the load on freshwater sources, microalgal species that can grow and reproduce in environments with moderate to high salinity seems to be good choice for biofuel production (Li et al. 2008). *D. viridis*, due to its high growth rate and its unique advantage of growth in coastal regions using sea water have been considered for biofuel production. In the present study, the effects of sodium nitrate and glucose in a mixotrophic culture on biomass and lipid content of *D. viridis* were investigated. In addition, the influence of different plant hormones on the growth of this microalga was evaluated.

MATERIAL AND METHODS

Microalgae

Dunaliella viridis Teod. was sampled from Maharlu salt lake located in southeast of shiraz (latitude 29.26 N, longitude 52.48 E), Iran. The alga was purified and identified on the basis of morphology and rDNA ITS sequences as previously described (Kharati-Koupaei et al. 2012). The sequences was submitted to NCBI gene bank under the accession number HQ864830.

Algal growth conditions

D. viridis was cultured in 250-mL flasks with 100 mL sterile growth medium containing 2.0 M NaCl, 50.0 mM $NaHCO_3$, 5.0 mM $MgSO_4$, 1.0 mM $NaNO_3$, 0.2 mM KH_2PO_4, 0.2 mM $CaCl_2$, 7.0 μM $MnCl_2$, 5.0 μM EDTA, 2.0 μM $FeCl_3$, 1.0 μM $CuCl_2$, 1.0 μM $CoCl_2$, 1.0 μM $(NH_4)_6Mo_7O_{24}$, and 1.0 μM $ZnCl_2$ (pH 7.5). The axenic cultures were incubated in a growth chamber at 22 ± 2ºC under light/dark regime 16/8 h.

Illumination was provided by cool white fluorescent lamps at an intensity of 135 μmol quanta $m^{-2}s^{-1}$. (Nikookar K et al. 2004; Zamani H et al. 2011). To test the effects of sodium nitrate different concentrations of sodium nitrate (0.0, 0.5, 1.0, 2.5, 5.0 and 10.0 mM) were added to the algal culture the cell number and lipid content were measured at indicated time intervals.

Mixotrophic culture and its effects on biomass and lipid content of the algal cells were studied by adding different concentrations of glucose (0.0, 5.0, 10.0, 15.0 and 20.0 g L^{-1}) to the cultured.

To investigate the effects of plant hormones, Indole-3- acetic acid (IAA) and Indole-3-butyric acid (IBA) were added to the culture media at final concentrations of 10^{-12}, 10^{-9}, 10^{-6} and 10^{-4} M IAA, or 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M IBA. To test the synergetic effects of IAA and trans- zeatin riboside, IAA at 10^{-9} M and different concentrations of trans -zeatin riboside (10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M) were added to the culture media. To evaluate the synergetic effects of IBA and kinetin, 10^{-6} M IBA and different concentration of kinetin (10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M) were added to the culture media and cell number was measured throughout the experiment.

Growth determination

To determine the number of cells in one mL of the algal suspension, samples were taken from the culture medium and absorbance at 680 nm was recorded using Shimadzu spectrophotometer model UV-160 A. The following formula was derived from the standard curve:

Number of cells per mL = $((OD_{680}-0.0164) / 9) \times 10^8$

Lipid measurment

Lipid content was determined according to the method of Izard and Limberger (Izard et al. 2003) with slight modification. Ten mL of algae culture was centrifuged for 10 min at 5000 g. The supernatant was discarded, and the pellet was washed once by the cell-free culture medium centrifuged. The supernatant was discarded and to the pellet was added 0.8 mL distilled water, 2.0 mL methanol and 1.0 mL chloroform and mixed thoroughly at 4°C. After addition of 1.0 mL chloroform and 1.0 mL distilled water, the mixture was mixed for 1.0 min. and centrifuged at 3000g for 15 min. The chloroform layer containing the lipids was separated and 100 µL of it was transferred to a test tube. The chloroform was evaporated using nitrogen gas and 2.0 mL of 18 M sulfuric acid was added to the test tube. The mixture was incubated in a boiling water bath for 10 min. After cooling and addition of 5.0 mL phosphoric acid-vanillin reagent, the tubes were incubated at 37°C for 15 min. The phosphoric acid–vanillin reagent was prepared by the addition of 0.60 gram of vanillin to 20 mL of distilled water and the volume was adjusted to100 mL with 85% phosphoric acid. The tubes were kept at room temperature and their absorbance were recorded at 530 nm using Shimadzu spectrophotometer model UV-160 A. The standard curve was constructed using Triolein in the range of 1.0 to 5.0 mg.

Statitical analysis

The analysis of data was performed using the SPSS 17 Software and all graphs were plotted with Excel 2007.

RESULTS

Effects of different concentrations of sodium nitrate (mM) on the growth and lipid production

As shown in Figure 1, increase in cell number per mL of culture was observed with elevation in sodium nitrate concentration up to 5.0 mM. At 10.0 mM sodium nitrate, growth rate was the same as that in the presence of 5.0 mM sodium nitrate. In the absence of sodium nitrate no increase in cell number was observed.

Table 1 shows the effects of different

concentration of sodium nitrate on growth and lipid content 20 days after the start of experiment which is essentially late logarithmic phase of growth. As stated before, growth increased with increase in sodium nitrate concentration. Lipid content expressed as µg per mL of culture media also was elevated with increase in sodium nitrate up to 5.0 mM. Higher concentration of sodium nitrate had no effect on lipid content. When total lipid was calculated on the cell basis, highest lipid content occurred at the lowest sodium nitrate concentration.

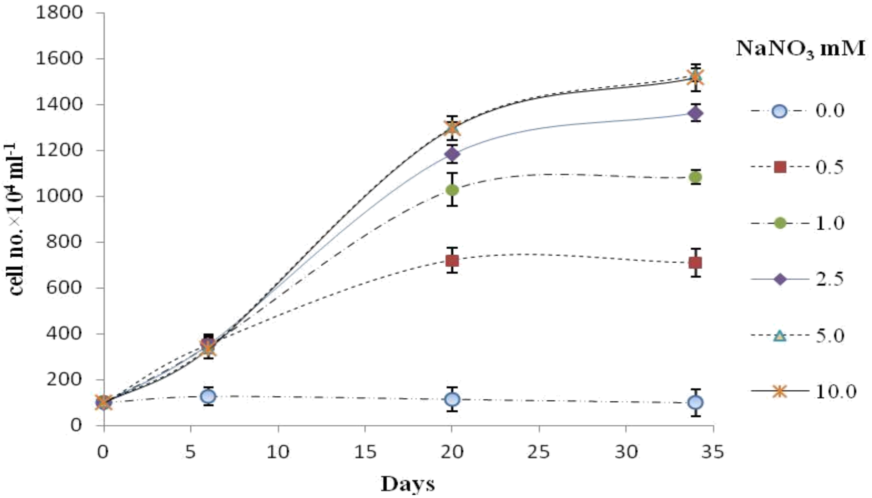

Figure 1- Growth of *D. viridis* strain MSV-1 at various concentrations of $NaNO_3$. Each number is mean ± SE

Table 1- Effects of different concentrations of $NaNO_3$ on cell number and total lipids content 20 days after the start of experiment.

	$NaNO_3$ (mM)				
	0.5	1.0	2.5	5.0	10.0
cell number ×10^4 mL^{-1}	720 ± 114 (70%)	1028 ± 53 (100%)	1182 ± 70 (115%)	1300 ± 80 (126.5%)	1296 ± 22 (126.1%)
total lipids (µg mL^{-1})	61.7 ± 4.7 (93.9%)	66.7 ± 10.3 (100%)	75 ± 7.1 (115.2%)	78.3 ± 10.3 (118.2%)	78.3 ± 10.3 (118.2%)
total lipids (pg $cell^{-1}$)	8.6 ± 0.12 (134.4%)	6.4 ± 0.1 (100%)	6.4 ± 0.09 (100%)	6.0 ± 0.02 (93.7%)	6.0 ± 0.01 (93.7%)

Each value is mean ± SE. Values in parenthesis are percentages relative to control (1 mM $NaNO_3$).

Effects of different concentrations of glucose on the growth and lipid production

To study the possible effect of mixotrophic culture on the growth and lipid production by *D. viridis*, glucose at concentrations of 5.0, 10.0, 15.0 and 20.0 g L^{-1} was added to algal culture containing 5.0 mM sodium nitrate (Fig. 2). During entire period of experiment, glucose at 5.0 g L^{-1} did not show significant difference compared to control ($P < 0.05$). At 10 g L^{-1}, glucose affected cell number significantly ($P < 0.05$) at late logarithmic and stationary growth phases. Growth at higher glucose concentration was comparable to 10 g L^{-1} glucose during the entire period of experiment except at day 35, which 20 g L^{-1} glucose caused highest cell production in the culture media (Fig. 2).

Lipid content per mL of culture augmented rapidly with increase in glucose concentration up to 10 g L^{-1} afterwhich only gradual increase in lipid content was observed. When total lipids was calculated on the cell basis and expressed as pg $cell^{-1}$ rapid increase in the cell lipid content occurred at 5.0 g L^{-1} glucose with relatively small changes at higher glucose concentration (Table 2).

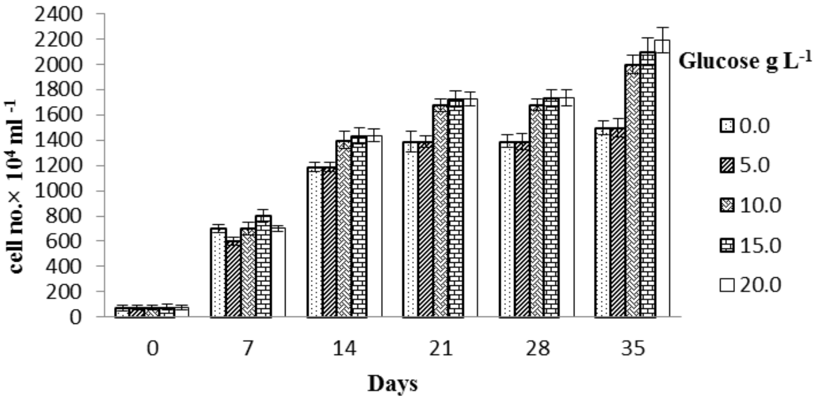

Figure 2- Effect of different concentration of glucose (mixotrophic culture) on the growth of *D. viridis.* Culture contained 5.0 mM sodium nitrate. Each number is mean ± SE.

Table 2- Effects of different concentrations of glucose (mixotrophic culture) on cell number per mL of culture and total lipids content (mg mL^{-1} and pg $cell^{-1}$). Sodium nitrate concentration was adjusted at 5 mM.

	Glucose concentrations (g L^{-1})				
	0.0	5.0	10.0	15.0	20.0
cell no.×10^4 mL^{-1}	1363 ± 47 (100%)	1345 ± 69 (103.4%)	1706 ± 52 (131.2%)	1770 ± 46 (136.2%)	1771 ± 69 (136.4%)
total lipids (µg mL^{-1})	78.3 ± 10 (100%)	135 ± 4 (173%)	173.3 ± 6 (224.3%)	178.3 ± 2.6 (229.7%)	181.7 ± 10.3 (235.1%)
total lipids (pg $cell^{-1}$)	6.0 ± 0.4 (100%)	10.0 ± 0.3 (166.7%)	10.2 ± 0.3 (170.0%)	10.1 ± 0.2 (168.3%)	10.3 ± 0.5 (171.7%)

Each number is mean ± SE. Values in parenthesis are percentages relative to control.

Effects of plant hormones on the growth of *Dunaliella viridis*

Indole acetic acid (IAA) at 10^{-12} and 10^{-9} M did not have any significant effect on algal growth. At 10^{-6} M IAA growth was reduced to 74.1 percent relative to control (Table 3). At higher concentration (10^{-4} M) algal growth was significantly reduced to 4.1 percent; i.e., 95.1 percent growth inhibition had occurred. Slight increase in algal cell number was observed in the presence of indole butyric acid (IBA). The highest but still insignificant increase in cell number occurred at 10^{-10} M IBA. In the presence of trans-zeatin riboside plus 10^{-9} M IAA increase in cell number was observed at all concentrations of trans-zeatin riboside tested, being significant at 10^{-8} and 10^{-6} M of this hormone. Kinetine plus 10^{-6} M IBA caused augmentation of growth at all tested concentration, but the increases were statistically insignificant at $P < 0.05$.

Table 3- Effects of IAA, IBA, trans-zeatin riboside and kinetine on the growth (cell number $\times 10^4$ mL^{-1}) of *D. viridis.* Sodium nitrate concentration was adjusted at 5 mM.

Hormones	Concentration (M)						
	0.0	10^{-12}	10^{-10}	10^{-9}	10^{-8}	10^{-6}	10^{-4}
IAA	1300 ± 24 (100%)	1304 ± 42 (100.3%)	----- -----	1300 ± 24 (100%)	----- -----	964 ±26 (74.1%) *	54 ± 24 (4.1%)*
IBA	1278 ± 50 (100%)	1310 ± 54 (102.5%)	1390 ± 48 (108.8%)	----- -----	1318 ± 34 (103.1%)	1300 ± 52 (101.7%)	1284 ± 32 (100.5%)
trans zeatin-riboside + 10^{-9} M IAA	1310 ± 54 (100%)	1512 ± 40 (115.4%)	1500 ± 46 (114.5%)	----- -----	1608 ± 32 (122.8%)*	1580 ± 22 (120.6%) *	----- -----
Kinetine + 10^{-6} M IBA	1300 ± 50 (100%)	1354 ± 42 (104.2%)	1392 ± 74 (107.1%)	----- -----	1314 ± 32 (101.1%)	1352 ± 52 (104.0%)	----- -----

Each number is mean ± SE. Values in parenthesis are growth percentages relative to control.
Stars show significant difference at $P < 0.05$.

DISCUSSION

The biomass and cell lipid content are the key parameters affecting the commercial biofuel production from microalgae (Chen et al. 2011). Although high biomass is obtained under optimal growth conditions, high cell lipid content is usually occurres under stresses such as nutrient deficiency (Hu et al. 2008). Nitrogen limitation is known to increase cell lipid content of many microalgae (Parrish and Wangersky 1987). Results for cell lipid content under nitrogen limitation in *Dunaliella* are rather controversial. Total lipids did not change significantly in *D. primolecta* (Uriarte et al. 1993) and *D. tertiolecta* (Lombardi and Wangersky 1995) under nitrogen deficiency. Similar results were reported by Gordillo et al. (1998) with respect to the cell total lipids in *D. viridis* under nitrogen limitation at high and atmospheric CO_2 levels. But, they showed that triacylglycerol increased from 0.98 to 21.5 percent under nitrogen limitation at one percent CO_2. In another study, Chen et al. (2011) showed time-dependent accumulation of lipids in *D. tertiolecta* under nitrogen starvation. *D. viridis* is a fast growing microalgae and as shown in the present study, increase in nitrate concentration augmented its biomass production. As with many microalgae (Nigam et al. 2011), due to increase in cell number, total lipids per mL of culture increased with elevated nitrogen concentration; total lipids per cell decreased slightly at high nitrate concentration, but at 0.5 mM nitrate 34.4 percent increase in cell lipid content was observed.

The mixotrophic growth occurs when some algae combine photosynthesis with heterotrophic assimilation of organic compounds such as glucose (Brennan and Owende 2010). When *Spirulina* sp. Growth in photoautotrophic, heterotrophic and mixotrophic cultures were compared, it was found that mixotrophic culture improved growth rate over both autotrophic and heterotrophic cultures (Chojnacka and Noworyta 2004). In *Chlorella vulgaris*, mixotrophic culture with 1.0 percent glucose showed the highest lipid productivity compared to other growth conditions (Liang et al. 2009). Abreu et al. (2012) showed that mixotrophic cultivation of *Chlorella vulgaris* had higher specific growth rate, final biomass production and also higher lipid productivity. The biomass and lipid production of tested strains in mixotrophic culture were higher compared with other methods of culture especially with marine *Chlorella* sp. And *Nannochloropsis* sp. (Cheirsilp and Torpee, 2012). Increasing light intensity and initial glucose concentration enhanced the growth of both strains but lipid content was reduced. In our experiment, increasing glucose concentration increased biomass and as a result lipid content per mL of culture. Total lipid per cell increased at 5.0 g L^{-1} of glucose and then showed small changes compared to control.

Essentially all known phytohormones are detected in various algal taxa and their biological activities are similar to their function in higher plants (Tarakhovskaya et al. 2007). Enhancement of *Scenedesmus obliquus* growth and lipid content in the presence of indol-3-acetic acid (IAA) was reported by Salama et al. (2014). Kinetin and 2,4-dichlorophenoxy acetic acid (2,4-D) caused significant increase in the growth of *D. salina* (de Jesus Raposo, M. F. and R. M. S. C. de Morais, 2013). In the present experiment, phytohormones did not have profound effect on the growth of *D. viridis.* It seems that right combinations of different phytohormones with proper concentrations may increase lipid productivity in microalgae.

CONCLUSION

Despite increase in lipid productivity (biomass plus cell lipid content) by mixotrophic cultivation compared to photoautotrophic cultures, less costly organic carbon sources must be searched for to reduce the cost of mixotrophic cultivation and thus, make commercial production of biofuels from microalgae economically feasible. Due to the high cost of phytohormones and their small effects on biomass production, their use in mixotrophic culture is not suggested unless combination of cheap synthetic hormones or their analogs with right concentrations give a significant increase in lipid productivity.

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Erratum

In the 01 page, that read:

“**Mansour Kharati-Koupaei[1]; Ali Moradshasi[1*]**”

Read:
“**Mansour Kharati-Koupaei[1]; Ali Moradshahi[1*]**”