Original Research

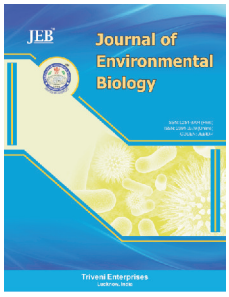
Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

DOI : http://doi.org/10.22438/jeb/39/5/MRN-761

JEB™

p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP



Modulation of lipid productivity under nitrogen, salinity and temperature stress in microalgae *Dunaliella* sp.

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Key words

Biomass production
Dunaliella sp.
Eicosapentaenoic acid
Lipid production
Physiological stress

Publication Info

Paper received : 29.09.2017
Revised received : 18.01.2018
Re-revised received : 18.03.2018
Accepted : 04.04.2018

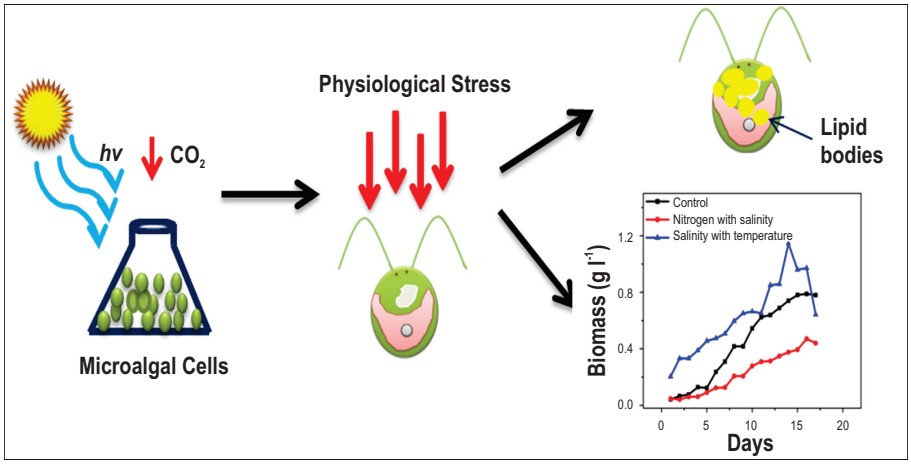
Abstract

Aim : Effect of physiological stress, namely, nitrogen depletion, salinity and temperature on the biomass productivity and lipid productivity in *Dunaliella sp.* was investigated.

Methodology : Culture conditions of *Dunaliella* sp. was optimized to maximise biomass productivity. Under this condition, it was subjected to two specific pattern of physiological stress, namely nitrogen depletion with increased salinity and increased salinity with temperature shock. The biomass and lipid productivities under these stress conditions were monitored. The extracted lipids were further characterized using gas chromatography - mass spectroscopy method.

Results : Laboratory culture conditions for *Dunaliella* sp. were optimized to yield maximized biomass productivity. It exhibited doubling of lipid content under nitrogen limitation with high salinity, though overall biomass yield under this stress pattern had significantly decreased. Characterization of the accumulated lipids showed a significant increase in the unsaturated fatty acid production. However, a noticeable decrease in saturated fatty acid was observed under the stress pattern of high salinity followed by temperature shock.

Interpretation : Algae biomass yield for *Dunaliella* sp. was maximum under laboratory conditions to which physiological stress was applied. It results in modulation of biomass and lipid productivity with a decrease in biomass and increase in lipid accumulation per unit dry biomass. Interestingly, it was found that such physiological perturbation in *Dunaliella* sp. result in modulation of lipid profile leading to increased accumulation of Omega 3 fatty acid.



Introduction

Microalgae are promising candidate to extract lipid oil. The fatty acid profiles of these microalgae lipids have been found to be suitable for biodiesel production (Gouveia and Oliveira, 2009). This has led to renewed interest in the development of cost effective algae culture for biodiesel production world over. The focus of research using microalgae has been to search for photoautotrophic strains that give the higher yield of lipid in short span (Converti *et al.,* 2009; Mata *et al.,* 2010). Identification of high yielding algal strain require characterization of their lipid and biomass productivity under various physiological conditions.

The process of increasing the yield of biomass and lipid in microalgae by administering physiological stress at the appropriate time of growth is well known. Stress in terms of nitrate (Li *et al.,* 2008; Yeesang and Cheirsilp, 2011; Martin *et al.,* 2014), phosphorous (Adenen *et al.,* 2016), light (Solovchenko *et al.,* 2008; Braun *et al.,* 2014; Sharma *et al.,* 2015), salinity (Takagi and Yoshida, 2006; Roleda *et al.,* 2013) and temperature (Renaud *et al.,* 2002; Converti *et al.,* 2009; Roleda *et al.,* 2013) at a particular state of its growth cycle have been considered to stimulate accumulation of lipids. These algae, which have the capability to accumulate lipids to the order of 50-70% under these conditions, also lead to the production of value-added chemicals (Guedes *et al.,* 2011) that can help to compensate and stabilize the economy of biofuel production process.

It has been shown that the fatty acid composition of polar lipids in *Dunaliella salina* was affected significantly by the change in salinity (Peeler *et al.,* 2017). In the case of *Boekelovia hooglandii,* the percentage of saturated fatty acid decreased as the concentration of NaCl increased, while the percentage of polyunsaturated fatty acid increased (Fujii *et al.,* 2001). Light and salinity play an important role in triggering particular physiological stage in microalgae cultures. On studying the effect of photons, it has been reported that 18:6 hrs light:dark cycles are optimal for microalgae growth (Braun *et al.,* 2014). Experimental and *in silico* studies among different algal species show the biomass content and lipid composition vary with culture and physiological conditions such as temperature, nutrient and light intensity (Converti *et al.,* 2009; Li *et al.,* 2008; Solovchenko *et al.,* 2008; Shah *et al.,* 2017). There are many instances where microalgal species have been reported to increase unsaturated fatty acids with decreasing growth temperature and increased saturated fatty acids with increasing growth temperature (Renaud *et al.,* 2002). *Chlorella* sp. and *Monodus subterraneus* have shown significant increase of lipids under nitrogen deficiency (Illman *et al.,* 2000; Khozin-Goldberg and Cohen, 2006) and *C. vulgaris* upon Fe^{3+} induction in the late log phase exhibited 3-7 fold accumulation of lipid (Liu *et al.,* 2008). Typically, nitrate stress has been found to influence the lipid yield where limiting nitrate increases the lipid content at the expense of biomass. Different nitrogen sources would affect the growth and lipid content of microalgae (Li *et al.,* 2008). Oil content of some microalgae such as *Scenedesmus* sp., *Chlorella* sp., *Neochloris oleoabundans* increased under stress from 20% to 50% of total cell dry weight, revealing the significant potential for biodiesel production. Thus, microalgae response to physiological perturbation found to be dependent on types of stress and also on the nature of species. Therefore in the present study, in order to explore an optimal growth conditions for lipid and biomass productivity in *Dunaliella* sp., effect of combination of physiological stress conditions, namely, nitrate limitation, temperature and high salinity was investigated. Fatty acid methyl esters of *Dunaliella* sp. derived under these conditions were further analyzed for its suitability for biofuel and potential value added chemicals.

Materials and Methods

Algal strains and culture conditions : Marine microalgae *Dunaliella* sp. was purchased from Central Marine Fisheries Research Institute (CMFRI), Tuticorin, Tamilnadu, India. The strain was cultured in Walne's media (sterilized seawater, supplemented with inorganic salts) under 18:6 hrs light: dark period. A white fluorescent light at 300 µmol m^{-2} s^{-1} photons provide required light exposure. Temperature was maintained at 23ºC and pH of the culture was set to 7.5.

Culturing under nitrogen, salinity and temperature stress conditions : *Dunaliella* sp was cultured under nitrogen limitation, high salinity and high-temperature stress conditions. The inoculum of *Dunaliella* cells were transferred to four 20 l cleaned and sterilized transparent polypropylene canes containing 9 l culturing medium. The cultures were aerated by 5 W aquarium pump to provide required mixing by airlift. Three culturing tanks were named as per culturing condition viz., control, nitrogen depletion with high salinity, and high salinity with temperature shock. The control culture was maintained in normal seawater based medium with salinity. In that the light and media were optimized to yield maximum biomass. For nitrogen depletion with high salinity stress pattern, the nitrogen-depleted media was prepared in similar fashion minus sodium nitrate. The nitrogen depleted medium (9l) at NaCl concentration 1 M above the sea water concentration was introduced at the early log phase (6^{th} day) so as to result in 0.5 M excess salt with 50% nitrogen depletion in the final culture. For stress pattern of high salinity with temperature shock, normal medium (9l) with added NaCl concentration at 1 M above the sea water concentration was introduced in the log phase (8^{th} day) so as to result in 0.5 M excess salt in the final culture which on the 13^{th} day, subjected to a temperature stress by elevating it to 28ºC from 23ºC. A 4 ml of sample was harvested on each day basis to determine the biomass growth rate and neutral lipid content.

Measurement of biomass growth : The growth of *Dunaliella* sp. was determined by measuring the optical density at 682 nm of each day samples by UV spectrophotometer. The biomass specific growth rate k was calculated by the following formula :

$$k = (\ln N - \ln N_0) / (t - t_0)$$

Where, k (d^{-1}) is the specific growth rate at exponential growth phase, N_0 is dry weight at the beginning of exponential phase (t_0) and N represents the dry weight at the time (t) of the exponential phase. The value of dry biomass was deduced from calibration curve of biomass vs O.D. determined for *Dunaliella* sp. (Data not shown). From the plot of dry biomass weight vs growth time, within exponential growth phase (Ma *et al.,* 2014) k was deduced from linear fit to the data. The biomass doubling time was calculated based on the specific growth rate. After 16 days of culturing, total biomass was harvested by electroflocculation. In order to prevent cell disruption, pH of the media during electroflocculation was maintained between 7.2 to 8.5. Collected biomass was centrifuged at 7000 rpm for 10 min in 6K15 Sigma laboratory centrifuge and washed twice with distilled water to remove adhering inorganic salts.

Lipid detection by FTIR : Dried *Dunaliella* sp. biomass powder was mixed with potassium bromide and pelletized as a tablet for the FTIR measurement. Functional groups in each stress and control samples were examined by Fourier Transform Infrared Spectrometry (FTIR) method. Fourier transform infrared spectra were obtained on a Thermo Nicolet-Model 6700 spectrometer with a scan range of 4000-450 cm^{-1}.

Estimation of total lipid : Quantification of total lipids was carried out by Sulphur–Phospho-Vanillin assay developed by Knight (Knight *et al.,* 1972) and adapted by Mishra (Mishra *et al.,* 2014) for algal strains. The standard lipid stocks were prepared by using refined sunflower oil dissolved in chloroform at a concentration of 2 mg ml^{-1}. To the different quantity chloroform-oil dispensed and dried in tube, 100 µl of deionized water followed by 2 ml of concentrated sulphuric acid were added and kept at 100ºC for 10 min and subsequently solution was cooled in ice bath for 5 min. 5 ml Phospho-vanillin reagent (200 ml of phosphoric acid, 0.3 g of vanillin, 5 ml of absolute ethanol and 45 ml of deionized water). was added to the samples mixed well for 30 min. The absorbance measured at 530 nm determine standard lipid curve. To determine the lipid content of given algal samples, a known volume (4 ml) of culture was centrifuged at 5000 rpm for 10 min and dried and SPV assay was performed as described above. *Dunaliella* sp. harvested on different days (5, 6, 8, 10, 14, 16 and 17th day) of growth were analysed for lipid content.

Analysis of fatty acid methyl esters (FAMEs) by GC-MS : For GC-MS studies, the total lipids from *Dunaliella* sp. grown under normal and two different stress conditions were extracted following modified Bligh and Dyer protocol (Yang *et al.,* 2014). Briefly, dry algal biomass was mixed in solvent containing chloroform: methanol (1:1, v/v) and subjected to ultra sonication for 30 min to break the cells. Then the reaction mixture was phase separated. Lipid was extracted from chloroform-rich phase in a rotary evaporator under vacuum condition. For GC-MS analysis, extracted lipid was mixed with n-hexane and sodium methoxide. The solution was vortexed vigorously for 10 min and stand to separate the layers. After 10 min the FAME containing clear solution was carefully separated from the cloudy aqueous phase and used for measurement.

The gas chromatography was performed in Thermo Trace ultra-Gas Chromatography system equipped with TSQ quantum Mass Spectrometry Detector. A 30 mm TR-5 Mass spectrometry column with 0.25 mm film thickness, composed of 5% phenyl, 95% methyl poly siloxane was used for sample run. The injection temperature was set at 250ºC, the ion source adjusted to 260ºC and the electron impact mode at 70 eV. Helium (99.99%) gas was used as a carrier gas at a constant flow rate of 1ml min^{-1}. The oven temperature was programmed to start at 50ºC (Isothermal for 0.5 min), with an increase of 40ºC min^{-1} to 175ºC (isothermal for 5 min), followed by 7 ºC min^{-1} to 225ºC (isothermal for 3 min) and 3ºC min^{-1} to 260ºC for 1min holding time. The retention time and mass spectra were identified and compared with fatty acid methyl esters of GC-MS library (NIST &WILEY).

Results and Discussion

Fig. 1 shows the normalized biomass growth curve for *Dunaliella* sp. cultured at 300 µmol $m^{-2}s^{-1}$ photons. The results shows the 18 l culture under normal, nitrogen depletion with high salinity and high salinity with temperature shock stress patterns yielded dry biomass of 0.78 gl^{-1}, 0.47 gl^{-1} and 0.64 gl^{-1}, respectively. It was observed that under normal grown (Table 1) the biomass productivity was higher compared to grown under stress conditions of nitrogen depletion with high salinity and high salinity with temperature shock. Limited availability of macronutrient, specifically nitrogen, hinders the biomass growth since it is one of the important macronutrient accounting>1 % of total dry biomass. It is known that, in general decrease in biomass growth rate during nitrogen difficiency off-sets the increase in lipid productivity leaving overal rates of oil production to be lower (Adams *et al.,* 2013; Minhas *et al.,* 2016). However, two fold higher biomass productivity was observed as compared to others (Ra *et al.,* 2015) under nitrogen stress.

The total lipid content was 0.0972, 0.1109 and 0.0314 g per gram dry biomass weight for control, nitrogen depletion with high salinity and high salinity with temperature shock conditions, respectively. The lipid content was found to drastically decrease under high salinity with temperature shock condition whereas it was enhanced under nitrogen depletion with high salinity condition. Taking into account the dry biomass yield per liter, a lipid fraction of 12.4%, 23.6% and 4.9% was observed for control, nitrogen depletion with high salinity and high salinity with temperature shock conditions (Table 1). Lipid productivity was calculated from the lipid content data derived from growth curve and is given in Table 1. The results revealed doubling of lipid accumulation under nitrogen depletion with high salinity condition. However, the decrease in biomass production under stress undermines the absolute lipid productivity to be lower compared to optimized culture. Whereas, under high salinity with temperature shock condition during log phase, it halved the biomass growth rate which was also evident from increase in the cell doubling time. Similar lipid fraction with increased biomass yield of 1.45 gl^{-1} has been reported for *Dunaliella salina* only under increased CO_2 level (Abd El Baky *et al.,* 2014). However, such

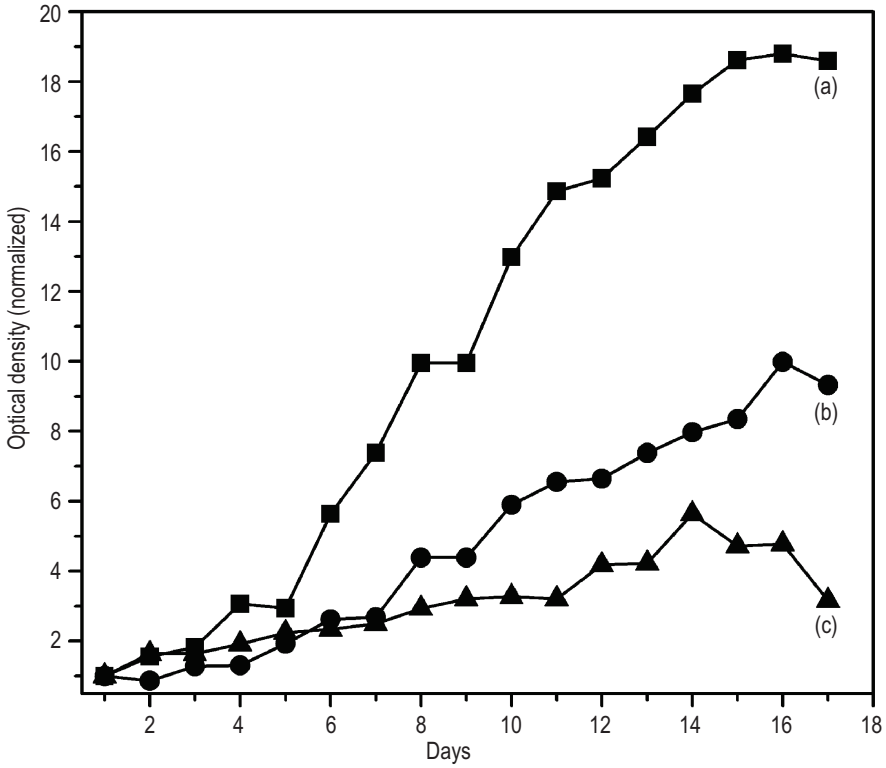


Fig. 1 : Growth of *Dunaliella* sp. under optimal condition (a, control) compared with nitrogen limiting with high salinity (b) and high salinity with temperature stress (c) conditions

efforts resulted in high total saturated fatty acid, about 85% compared to 35% in this study. For *Dunaliella salina* harvested during early stationary phase (Fakhry and Maghraby, 2013) similar lipid fraction was obtained but with a significantly lower biomass yield of 0.026 gl^{-1} with total unsaturated fatty acid of 64%. It is noted that the current approach resulted in 23.6% dry weight of lipid having 65% unsaturated fatty acid content (Table 2) but with significantly higher biomass yield of 0.47gl^{-1} under nitrogen stress (Table 1).

FTIR spectra of dry biomass of *Dunaliella* sp., given in Fig. 2, reveal eleven important absorption peaks corresponding to different organic compounds. The bands were assigned as a different vibrational mode. The absorption peak 2921 cm^{-1} asymmetric stretch and 2850 cm^{-1} symmetric stretch indicates the presence of (C-H) alkane group mainly fatty acids. The band 2362 cm^{-1} appearing asymmetrically indicates the presence of (P-H) phosphorous compounds. The two peaks appearing asymmetrically at 1743 and 1727 cm^{-1} were due to the vibrational stretching absorption of (C=O) ester group, primarily fatty acids and aldehyde group, respectively. The amine group (N-H) was characterized by the appearance of stretching band occurring at 1648 cm^{-1} and the asymmetrical stretch bend 1465 cm^{-1} indicated the presence of (CH_2) methylene group. The protein band spectra were observed as a (P=O) phosphodiester at the 1238 and 1195 cm^{-1} stretching vibrations. The carbohydrate

Table 1 : Analysis of biomass and lipid growth in *Dunaliella* sp. under optimal condition (control); nitrogen high salinity; salinity and temperature stress

Growth condition	Specific growth rate (d^{-1})	Doubling time (d)	Dry weight ($g\ l^{-1}$)	BP ($g\ l^{-1}d^{-1}$)	Lipid productivity* ($g\ l^{-1}\ d^{-1}$)	lipid yield ($g\ l^{-1}$)	Lipid (%)
Optimal growth	0.149 ± 0.016	4.65 ± 0.50	0.78	0.1305 ± 0.0094	0.0119	0.0972	12.43
Nitrogen limiting with high salinity	0.157 ± 0.019	4.41 ± 0.54	0.47	0.0683 ± 0.0061	0.0080	0.1109	23.60
High salinity and temperature stress	0.083 ± 0.009	8.34 ± 0.51	0.64	0.0717 ± 0.0092	0.0029	0.0314	4.90

*Lipid productivity estimated in the log phase of growth, precisely during 10^{th} to 16^{th} Day

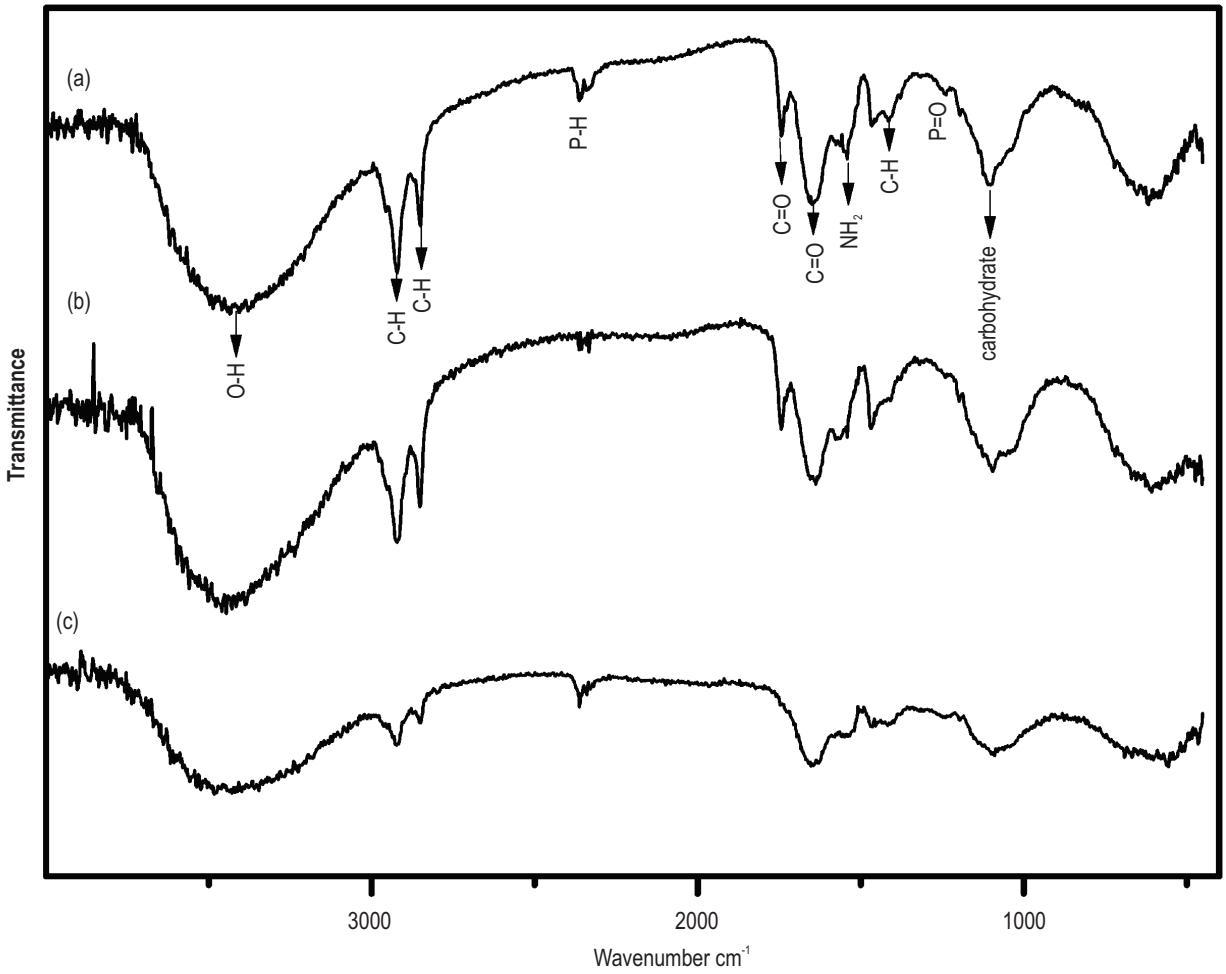


Fig. 2 : FTIR spectra for dry biomass of *Dunaliella* sp. cultured under (a) control (optimal condition); (b) nitrogen limiting with high salinity and (c) temperature stress with high salinity stress conditions

spectrum was characterized by the occurrence of symmetric vibrations at 1099 cm^{-1}. The O-H group of water was characterized by a strong band at 3407 cm^{-1}. The two absorption peaks of 2921 cm^{-1} and 2850 cm^{-1} indicated the presence of lipids, and were observed under both stress conditions including control *Dunaliella* sp. The C=O ester group (mainly lipids) indicating vibrational band 1743 cm^{-1} was also strongly observed under nitrogen depletion with high salinity pattern of stress giving evidence to higher lipid accumulation lipid as compared to control.

The results of fatty acid profiles of *Dunaliella* sp. cultured under normal condition showed (Table 2) the formation of major fatty acids namely, *myristic acid* (14:0), *palmitic acid* (C16:0), *palmitoleic acid* (C16:1) and *oleic acid* (C18:1). It was observed that under nitrogen depletion with high salinity stress condition, there was a two fold increase in total lipid content resulting in 23.6% dry weight. Under both control and nitrogen depletion with high salinity conditions major fatty acids were present. Notable among them was 5, 8, 11, 14, 17-Eicosapentaenoic acid an omega 3 fatty acid that was not present in *Dunaliella* sp. cultured under normal growth condition. The results of the present study compare well with all four species of *Dunaliella* reported in the literature (Ra *et al.,* 2015). However, it is emphasized that the present study could return 0.47 gl^{-1} biomass yield under these conditions compared to 0.28 gl^{-1} reported in literature (Ra *et al.*, 2015). From the analysis of lipid content, it was identified that palmitic acid (C16:0) was a major fatty acid under high salinity with temperature shock stress followed by oleic acid (C18:1), myristic acid (14:0) and Eicosapentaenoic acid, respectively (Table 2). Eicosapentaenoic acid expressed significantly under stress condition (Fig. 3a). Further analysis showed (Fig. 3b) 6.21, 12.56, 1.80% saturated 3.28, 8.96 and 1.17% unsaturated fatty acids in dry biomass under normal, nitrogen depletion with high salinity and high salinity with temperature shock stress conditions. Further, it was found that nitrogen depletion with high salinity stress leads to two fold increase in the saturated fatty acids, and about three fold increase in the unsaturated fatty acids content as compared to control. Whereas, there was a drastic decrease in total fatty acid, both saturated and unsaturated, of *Dunaliella* sp., under high salinity with temperature shock stress.

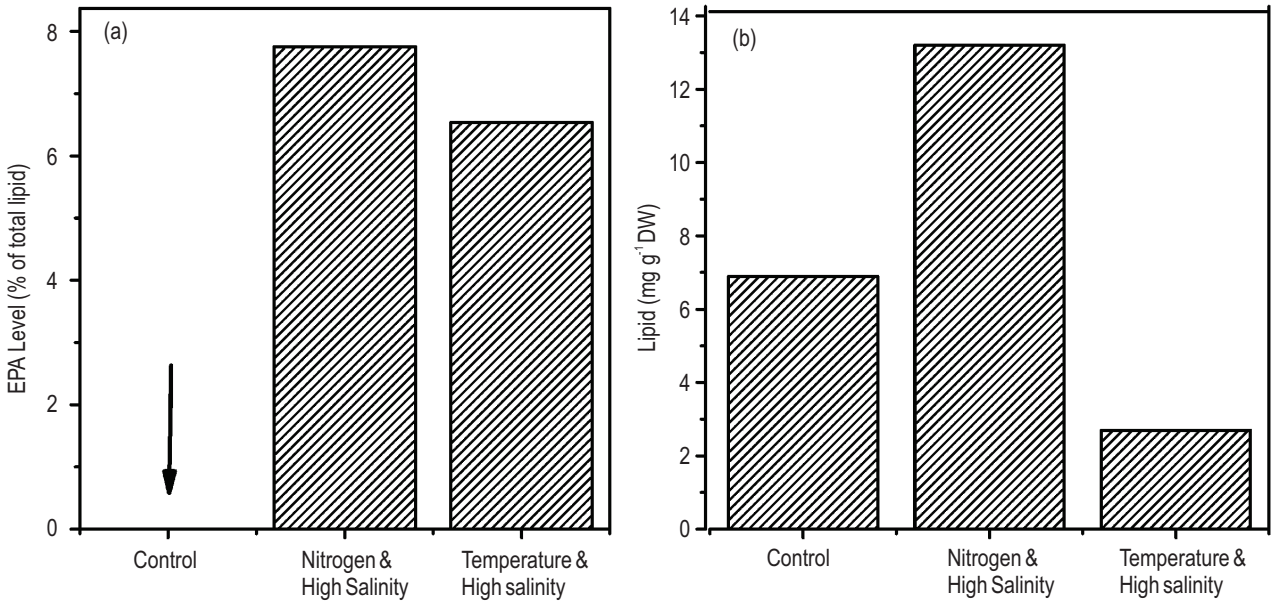


Fig. 3 : Distribution of lipids in *Dunaliella* sp. under stress: (a) Eicosapentaenoic acid (%) in total lipid and (b) saturated lipids (%) in dry biomass

The physiological perturbation had a significant effect on the formation of unsaturated chains of fatty acids. Specifically, it was observed that palmitic (C16:0) and palmitioleic (C16:1) are the major compounds produced regardless of nature of stress and culture condition (Table 2). It is noted that higher the unsaturated fatty acid, poorer is the oxidative stability of the derived biodiesel. However, unsaturated fatty acids also renders superior fuel property in terms of lower freezing point. We have observed optimal value of relative fatty acid content in for *Dunaliella* sp. to be 35% compared to 85% (Fakhry and Maghraby, 2013) and 15% (Abd El Baky *et al.,* 2014) reported in the literature.

It was observed that growth under nitrogen depletion with high salinity stress in *Dunaliella* sp. resulted in higher levels of C16-C18 fatty acids (Table 2), including C16:0 (palmitic acid), C16:1 and C18:1 (oleic acid) that are classified to be ideal feedstock for biodiesel production (Islam *et al.,* 2013). In addition, poly unsaturated omega 3 fatty acid EPA having great benefits to human health (Guedes *et al.,* 2011; Kent *et al.,* 2015) expressed significantly under nitrogen depletion with high salinity and high salinity with temperature shock stress conditions. This analysis revealed that resulting fatty acid composition is specific to growth condition, and thus can probably influence selection of particular growth condition for biodiesel or food industry application.

For biodiesel application, FAMEs such as C16:1, C18:1 are desirable feedstock. It is noted that both of these FAMEs were present in higher percent in the lipid extract, regardless of the type

Table 2 : The fatty acid profile of *Dunaliella* sp. cultured under optimal growth (control), nitrogen limiting with high salinity (NS) and high salinity with temperature stress (ST) conditions

		FAME (%)		
FAME	**Name**	**Control**	**NS**	**ST**
C14:0	Myristic acid	8.49	0.70	-
C15:0	Methyl myristate	-	6.38	8.54
C16:0	Palmitic acid	40.09	44.72	28.22
C17:0	15-methyl palmitic acid	1.41	-	-
C18:0	Stearic acid	0.52	-	
C19:0	Methyl stearate	-	1.41	-
C20:2	11,14-Eicosadienoic acid	-	0.89	-
C16:1	Palmitoleic acid	16.91	18.78	-
C18:1	Oleic acid	9.49	10.57	17.35
C20:5	EPA	-	7.75	6.54
Total saturated		50.51	53.21	36.76
Total unsaturated		26.4	37.99	23.89
Total FAME		76.91	91.2	60.65

stress conditions. This property makes *Dunaliella* sp. an important candidate for biodiesel industry. Further, co-production of omega-3 fatty acid such as EPA, an important product beneficial for human consumption, can significantly compensate the higher production costs of biomass from microalgae. It is emphasized that for the above reason, there have been specific attempt to increase the production of Omega-3 fatty acids and carotene in *Dunaliella* sp., wherein CO_2 supplemented growth conditions has been tried (Chagas *et al.,* 2015). However, in the present study, we observe significant production of Omega-3 fatty acid was observed under specific stress conditions.

Dunaliella sp. is one of the promising candidate microalgae for developing integrated approach in biofuel production. It has been reported that optimized normal growth of *Dunaliella* sp. under batch culture yielded 0.78 gl^{-1} biomass. Under physiological stress, though cell biomass decreased, two-fold increase in lipid content and modulation of lipid composition was observed. Specifically, analysis of fatty acid profile under physiological stress revealed tuning of saturated and unsaturated fatty acids content and in that Omega-3 fatty acid being significantly modulated under stress. It is pertinent to note that *Dunaliella* sp. is known to yield increased amount of β-carotene under combined light and nitrogen stress (Markou and Nerantzis, 2013) and to a significant level under optimal growth condition (Kent *et al.,* 2015). This further corroborates the need to explore different stress pattern to derive optimal lipid and biomass productivity which simultaneously results in enhanced production of value added chemicals.

Acknowledgments

We acknowledge SMS Labs Services, Chennai for the GC measurement, Dr.Hannah Rachel Vasanthi and Mr.Veeresh Kumar of Department of Biotechnology, Pondicherry University for their help and fruitful discussions, Ms.K. Alamelu for her technical support with FTIR data. AS acknowledges UGC-RGNF and ARS acknowledge UGC-MANF for financial support.

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