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Enhancing biomass and lipid production in marine microalga *Dunaliella tertiolecta* with physico-chemical modulation and using cheap fertilizer substrate

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ABSTRACT

Dunaliella tertiolecta, a marine microalga grows luxuriantly under saline conditions. In the present study, growth rate and lipid productivity of *Dunaliella tertiolecta* was increased by metabolic engineering and culture conditions using one variable at a time (OVAT). The culture was grown under different concentrations of nutrients like nitrogen (KNO_3), phosphorus (KH_2PO_4) and Sea salt with varied temperatures (28°C and 35°C) and light intensity. The maximum growth rate and lipid productivity of 11.52 mg/l/day and 10.323 mg/l/ day was obtained when *Dunaliella tertiolecta* was grown with 0.32 g/l KNO_3 and 0.2 g/l KH_2PO_4, respectively, keeping other resources constant. The highest lipid productivity of 15.33 mg/l/day was obtained at 36.5 g/l of sea salt concentration. The main objectives of the present study were to analyse and replace the culture media constituents with cheaper and cost effective components. We report the use of fertilizer, Suphala, as the cheap and readily available source for replacing nutrients like nitrogen (KNO_3), phosphorus (KH_2PO_4) and potassium in the modified culture media. Hence, it is a better option for large scale cultivation with the focus for enhanced biomass, lipid and biomolecule (carotenoid) production from *Dunaliella tertiolecta.*

Key words : *Dunaliella tertiolecta, Cheap fertilizer substrate, Marine algae*

Introduction

Dunaliella tertiolecta, a marine microalga has variegated utility in the biochemical industry owing to its high biomass and lipid content under saline conditions. There is a vast diversity of microalgae thriving under different environmental conditions. They are the rich source of antioxidants, pigments (carotenoids etc), vitamins and inorganic salts (Brown *et al*., 1999; Lubián *et al*., 2000). The use of microalgae for producing biodiesel is well known and is indispensable. Other uses include application in the pharmaceutical industry, biodiesel production, wastewater management, as nutritional supplements for human nutrition and as feed for animals and fish (Pulz and Gross, 2004).

For this very reason microalgae are grown in different culture media optimum for its growth. Over the years, numerous studies on microalgae cultivation is conducted with an aim to increase biomass production (Lee *et al*., 1996; Bhatnagar *et al*., 2011; Li *et al*., 2012) and there are several factors that determine the overall growth and concentration of biomass production and hence, eventually the protein, lipid and carotene content (Metsoviti *et al*., 2019). These factors can be categorized as physical and biological which include light, temperature, carbon dioxide concentration, pH, light intensity etc. (Wijffels

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et al., 2010). A plethora of studies are available focusing on optimization of algal growth and its productivity by alternating the concentration of inorganic nutrients such as phosphate (Gibor, 1956; Sukenik and Shelef, 1984), sulfate (Tafreshi and Shariati, 2009), CO2 (García-González *et al*., 2003) and bicarbonate (Hejazi *et al*., 2003), culture parameters such as temperature (Ben-Amotz,1995), pH (Ben-Amotz, 1995) and light intensity (Ben-Amotz and Avron, 1990).

The aim of this study is to use biochemical engineering for high growth rate and lipid production in *Dunaliella tertiolecta* under different concentrations of nutrients, salt, temperature and light intensity for its applicability at larger scale. Further, an attempt has been made to use cheap and easily available alternative sources as replacement for these nutrients.

Materials and Methods

Algal source, culture medium and inoculum preparation

The marine strain (*Dunaliella tertiolecta*) was procured from culture collection of The Energy and Resources Institute (TERI, New Delhi). The growth of microalga was optimized using two different media *viz.* f/2 media and Seawater medium (SWM). The f/2 medium (Guillardand Ryther, 1962) had a composition of (per litre) 33.6 g artificial seawater salts (Ultra Marine Synthetica Sea Salt, Waterlife), 75 mg $NaNO_3$, 5.65 mg NaH_2PO_4. $2H_2O$, 1 ml trace elements stock and 1 ml vitamin mix stock. The trace elemental solution (per litre) includes 4.16 g Na_2EDTA, 3.15 g $FeCl_3.6H_2O$, 0.18 g $MnCl_2.4H_2O$, 10 mg $CoCl_2.6H_2O$, 10 mg $CuSO_4.5H_2O$, 22 mg $ZnSO_4.7H_2O$, 6 mg $Na_2MoO_4.2H_2O$. The vitamin mix solution (per litre) includes 100 mg vitamin B1, 0.5 mg vitamin B12 and 0.5 mg biotin. Whereas, following was the composition of SWM media (Hirooka *et al*., 2020) *viz*., KNO_3 (0.5 g/l), KH_2PO_4 (0.2 g/l), $FeCl_3$ (0.0015 g/l), Sea salt (37 g/l) and enriched with 2 ml/l trace metal stock which is combination of EDTA (400 mg/l); $ZnCl_2$ (10mg/l); H_3BO_3 (150 mg/l); $CoCl_2.6H_2O$ (3.75 mg/l); $CuCl_2$ (10 mg/l). *D. tertiolecta* was cultivated at 26±2 °C using white LEDs with 50 µmol photons m -2 s -1 in culture room with light: dark regime of 16:8 h.

Evaluation of the microalgae growth: For evaluating the growth of *D. tertiolecta*, calibration curves (absorbance at 680 nm versus dry weight biomass concentration) was prepared. The experiment was performed in 200 ml cultures in triplicate by inoculating 14 days old culture. An absorbance measurement in a UV-Vis spectrophotometer at 680 nm and simultaneously dry weight were recorded daily. For the dry weight calculation, 25 ml culture was centrifuged at 5000 rpm for 15 minutes in a pre-weighed centrifuge tube and then drying up of algal cells in oven at 60 ºC till a constant weight was obtained.

Determination of microalgae biomass, lipids extraction and quantification

Microalgae biomass determination and lipid extraction was carried out at intervals of 7, 14, 21 and 28 days. Algal biomass was estimated by determining cell dry weight as described earlier. Total lipids extraction and quantification was performed as given by Bligh and Dyer (1959) method. The definite biomass sample obtained by centrifugation was weighed in a pre-weighed glass tube. Three solvents at ratios of 1, 2 and 0.8 (v/v) for chloroform, methanol, and distilled water, respectively were added. Subsequently, a second extraction step was performed by adding the co-solvents at ratios of 2, 2 and 1.8 (v/v) for chloroform, methanol, and distilled water, respectively. After centrifugation, three layers were obtained, i.e. an upper layer rich in water and methanol, a middle layer consisting of extracted biomass, and a lower layer rich in lipids and chloroform was obtained. The upper layer was discarded and the lower layer rich in lipids and chloroform was recovered to a previously weighed glass tube. The chloroform was evaporated to dryness and the purified lipids extract remained in the glass tube. The tube containing the lipids (pre-weighed when empty) was finally weighed again in order to determine the microalgae lipid content by gravimetry.

Biochemical engineering for high growth rate and lipid production: Algal growth and lipid production based on one variable at a time (OVAT) was performed to optimize and maximize growth and lipid production. Firstly, nutrient concentration was taken as variable for *D. tertiolecta*, which was cultivated in the standard medium with the modified concentration of Nitrogen and Phosphorous *viz*., Nitrogen: 25 mg l-1, 50mg L-1, 75 mg l-1 and Phosphorus: 2 mg l-1, 3 mg l-1, 5 mg l-1. Further nitrogen, phosphorus and potassium were replaced by cheap fertilizer sources (Suphala; NPK 19:19:19) and their concentration was optimized to enhance biomass and lipid productivity. The salt (NaCl) concentrations was also opti-

mized wherein microalga was grown in media with following salt concentrations viz., 0.6, 0.9 and 1.2 M. Microalgae was also grown under different intensity of light (35,55, and 100ìmol photon m-2.s-1) and temperature *viz*. 25, 30, 35 °C.

Growth and lipid production in D. tertiolecta using two stage processes: *D. tertiolecta* was initially grown using normal medium condition and then subjected to stress (salt or nutrient) condition. The time to introduce stress was optimized and its effect on lipid productivity was observed subsequently.

Results

Microalgal growth in two different cultivation media

D. tertiolecta growth was evaluated by growing it under two different media compositions. The growth pattern from 0 days to 35 days was recorded. An exponential growth pattern was observed under both media. But maximum biomass and lipid production in both the media (SWM and f/2) was recorded at the 14th day of algal growth (Fig. 1).

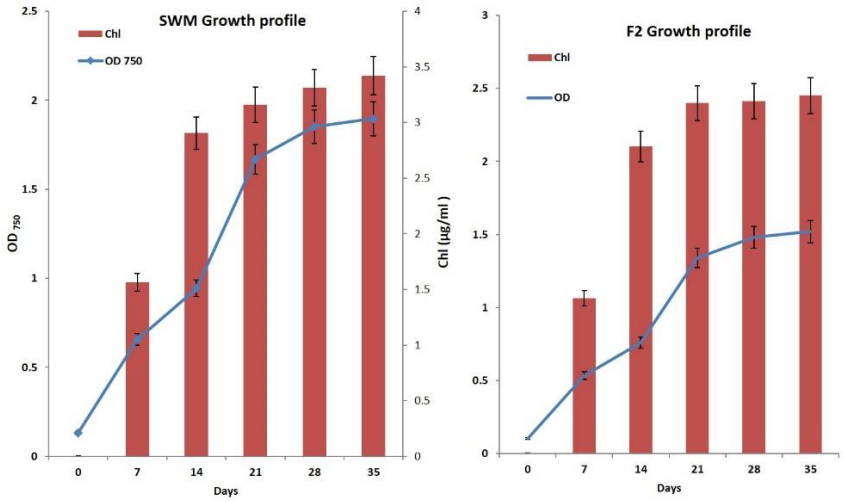

Fig. 1a. Comparison of *Dunaliella tertiolecta* for growth profile in different media viz., SWM and f2

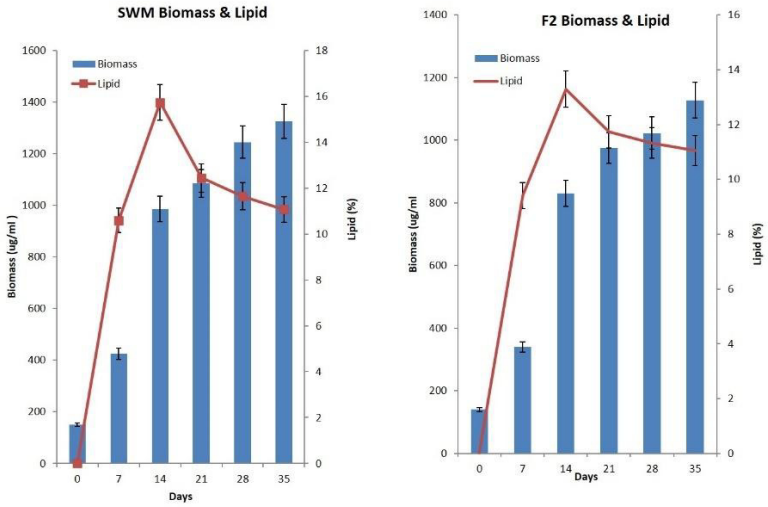

Fig. 1b. Comparison of biomass production and lipid productivity of *D. tertiolecta* for grown under two different media viz., SWM and f2

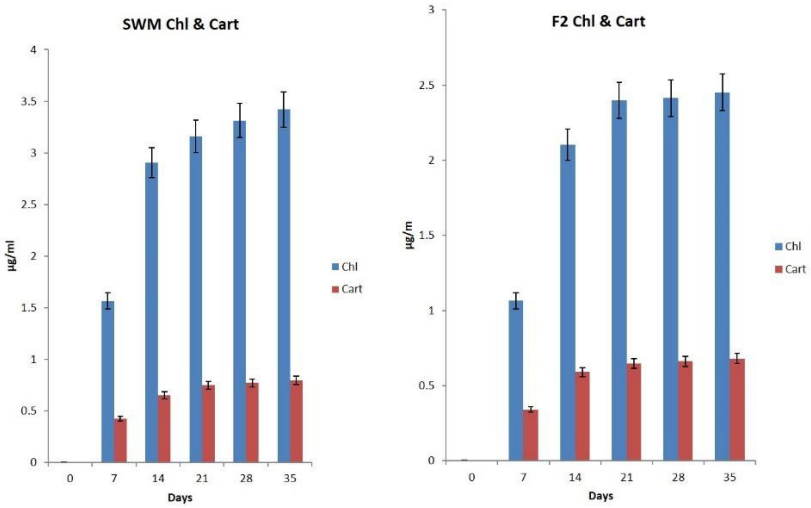

Fig. 1c. Comparison of chlorophyll and carotenoid content of *D. tertiolecta* when grown under two different media viz., SWM and f2

The growth was estimated by recording the absorbance (as mentioned in materials and methods) on every interval. Figure 1 shows the growth pattern of *D. tertiolecta* compared between the two media. The biomass production, lipid productivity and chlorophyll content.

Biochemical engineering for obtaining high growth rate and lipid production: One variable at a time analysis aided in determining the factors leading to higher biomass and lipid production. Nutrients play a vital role in determining the growth rate of any organism. Hence, the variable analysis was conducted by firstly analyzing the growth under different nutrient concentrations. The variable nutrients were nitrogen and phosphorus. Firstly, nitrogen (KNO_3) was the variable with rest of the nutrients as constants. As the concentration of nitrogen increased, there was a marginal increase noticed in the lipid and biomass production, however, a lower concentration of KNO_3 (0.32 g/l) was found to be optimum for maxi-

Table 1. Growth and lipid production studied under varied Nitrogen (a) and Phosphorus (b) concentrations for Biomass/Lipid productivity using one variable at a time (OVAT) approach

a)

Sea Salt (g/l)	KNO_5(g/l)	KH_2PO_4 (g/l)	Biomass (mg/ml)	Biomass Productivity (mg/l/Day)	Lipid (%)	Lipid Productivity (mg/l/Day)
36.5	0.64	0.4	1175	0.084	12.21	10.2
36.5	0.48(C)	0.4	1012.96	0.073	14.73	10.31
36.5	**0.32**	0.4	984.72	0.070	**16.18**	**11.52**
	CD		3.118	0.004	0.997	0.185
	CV		0.145	2.286	3.424	0.849

b)

Sea Salt (g/l)	KNO_5(g/l)	KH_2PO_4 (g/l)	Biomass (mg/ml)	Biomass Productivity (mg/l/Day)	Lipid (%)	Lipid Productivity (mg/l/Day)
36.5	0.48	0.6	1125.00	0.08	11.93	9.59
36.5	0.48	0.4(C)	989.00	0.071	13.54	9.57
36.5	0.48	**0.2**	985.08	0.07	**15.39**	**10.323**
	CD		2.373	0.004	1.006	0.468
	CV		0.113	2.351	3.676	2.338

mum lipid productivity. Furthermore, a similar trend was obtained in case of varied concentrations of phosphorus (KH_2PO_4). Phosphorus at its lowest concentration i.e., 0.2 g/l was found to be detrimental in growth rate of *D. tertiolecta* (Table 1a & b; Figure 2 a & b). This may be because the available phosphorus got exhausted and the organism was not able to survive under limited P availability.

***Effect of salt on D. tertiolecta growth*:** Since D. *tertiolecta* is a marine microalga, it was hypothesized that salt concentration will be one of the most important factors for its growth. For this, NaCl was used in the culture media at varied concentrations. The analysis revealed that even though *D. tertiolecta* thrives under salt conditions, the maximum growth and lipid productivity was not under the influence of high salt concentrations. 36.5 g/l concentrations of NaCl was found to be optimum to achieve highest biomass as well lipid production. Table 2 and Figure 3 shows the overall growth rate and effect of different salt concentrations on the growth of microalga.

Modified media, fertilizer use for optimum cheap microalgae growth: To further investigate the growth pattern of *D. tertiolecta* under different media composition. Source of nutrients, nitrogen phosphorus, and potassium was replaced by a common available fertilizer *viz*., Suphala with NPK in the ratio 19:19:19. It was interesting to note that this cheap nutrient source elevated the biomass production and lipid productivity. With the optimum media, the lipid productivity was 13.02 mg/l/day whereas, the modified media with fertilizer resulted in lipid production of 14.34 mg/l/ day (Table 3 a & b; Figure 4). Hence, a modified SWM media with a cheap source of fertilizer can be used in further applications pertaining to growth of *D. tertiolecta*.

Temperature affecting the growth of microalga: Not only the nutrients and temperature are also limiting factors in determining the maximum growth for an algae. This was further experimentally validated when *D. tertiolecta* was grown under two different temperatures in modified media (Table 4). 28°C was found to be the optimum temperature for its growth.

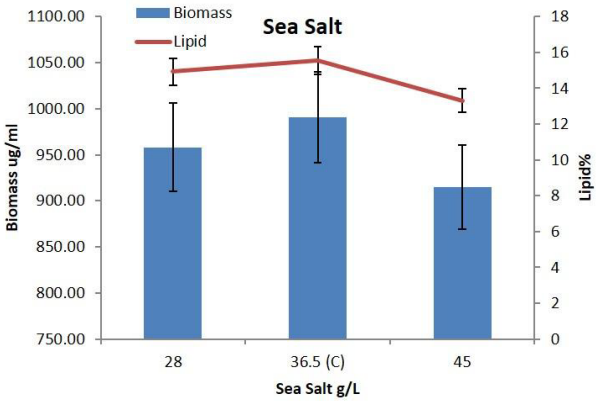

Fig. 3. Comparison of *D. tertiolecta* for Biomass and Lipid production under varied salt concentrations

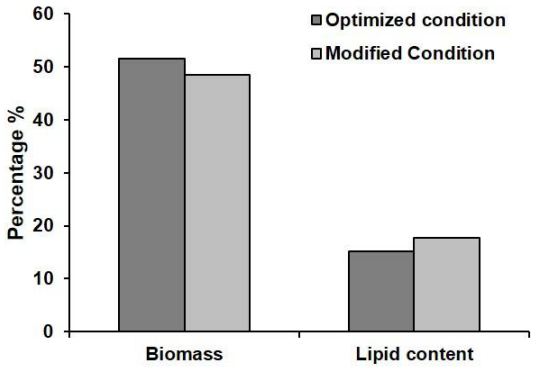

Fig. 4. Comparison of *D. tertiolecta* for Biomass and Lipid production at optimized and modified SWM media

Table 2. Growth and lipid production studied under varied salt concentrations for Biomass/Lipid productivity using one variable at a time (OVAT) approach

Sea Salt (g/l)	KNO_5(g/l)	KH_2PO_4 (g/l)	Biomass (mg/ml)	Biomass Productivity (mg/l/Day)	Lipid (%)	Lipid Productivity (mg/l/Day)
28	0.48	0.4	958.003	0.068	14.91	10.21
36.5(C)	0.48	0.4	990.623	0.065	**15.53**	**10.33**
45	0.48	0.4	915.01	0.065	13.31	9.13
	CD		3.103	0.002	0.919	0.374
	CV		0.16	1.774	3.093	1.858

Table 3. Nitrogen, Phosphorus and Potassium replaced by cheap fertilizer source (Suphala NPK 19:19:19) in modified media

a)

Factors	Unit	Optimum Condition	Modified Condition
Biomass	µg/ml	11970.75	11.27.43
Biomass Productivity	g/l/Day	0.086	0.081
Lipid content	%	15.21	17.80
Lipid Productivity	mg/l/Day	**13.02**	**14.34**

b)

Factors	Unit	Optimum Concentration	Modified Concentration
N(KNO_3)	g/l^{-1}	0.32	-
P (KH_2PO_4)	g/l^{-1}	0.2	-
NPK	mg l^{-1}	-	**220**
Fe ($FeCl_3$)	mg l^{-1}	1.5	1.5
Minor nutrient	ml l^{-1}	2	2
Sea Salt	g l^{-1}	36.5	36.5
Temperature	°C	±28	±28

Table 4. Biomass and lipid productivity in modified medium at two different temperatures (28f˜ C and 35 f˜ C)

Factors	Unit	28° C	35° C
Biomass	µg/ml	1149.18	1008.52
Biomass Productivity	g/L/Day	0.082	0.073
Lipid content	%	17.29	15.68
Lipid Productivity	mg/L/Day	14.19	11.29

Discussion

Dunaliella, a green alga, has adapted to strive under high salt concentrations (Ben-Amtoz and Avron, 1990). Its growth depends on complicated interactions of variables like nutrients, salinity, temperatures, and light. The present study has aimed to understand the growth pattern of *D. tertiolecta* under the effect of above-mentioned variables using two different media. Increased biomass and lipid productivity were the parameters to determine this effect. Out of two media, modified SWM media was found to increase the biomass and lipid production at the 14th day of its culture. Although Guillard f/2 media is a well-established culture media used regularly in industry, however, the overall growth profile for *D. tertiolecta* used in this study was much better with time when calculated using OD and the lipid productivity was also found to be maximum when cultured in SWM media.

It was further illustrated that biochemical engineering is a good tool for obtaining the optimum growth profile of microalgae. The one variable at a time (OVAT) approach revealed that although both the nutrients, i.e. nitrogen (KNO_3) and phosphorus (KH_2PO_4) are determinants for maximum growth; it is the nitrogen concentration of 0.32 g/l that resulted in maximum increase in the lipid productivity of 11.52 mg/l/day.

The effect of varied salt concentration on its growth has shown that *D. tertiolecta* can grow maximum up to a certain salt concentration and mere increase in salt concentration will not necessarily affect its growth positively. A 36.5 g/l of sea salt resulted in 15.53% of lipid production in comparison to 45 g/l of sea salt that resulted in only 13.31 % of lipid. There is a gamut of reports that have studied the effect of salt concentrations on *D. tertiolecta*. One such study is on *D. tertiolecta* (marine) and *D. viridis* (halophilic) wherein, it has been shown that *D. tertiolecta* grows faster under high salt condition but only up to a concentration of 3.5 M (Borowitzka *et al*., 1977, 1992; Rizwan *et al*., 2020). Another most studied species is *D. viridis* that is grown for its higher chlorophyll and carotenoid content (Ilknur *et al*., 2008). It has been reported that when D. viridis when grown in a medium with different salt concentrations (1, 2 and 3M), it showed an increase in its growth profile at 2 M concentration of NaCl (Ilknur *et al*., 2008). Another study reported 1M NaCl for the optimum growth of *D. viridis* (Jimenez and Niell, 1991). In the present study, higher lipid and biomass productivity was achieved at 36.5 mg/l salt concentration and higher concentration resulted in reduc-

tion of these parameters. Apart from salt and other nutrients, temperature also has an effect on growth of microalga. Our observations revealed that at 28 °C, a 2% increase in lipid productivity was observed as compared to 35 °C growth temperature. The results are in agreement with the findings of previous reports (Jimenez and Niell, 1991; (Ilknur *et al*., 2008).

In the present study, the growth of *D. tertiolecta* was evaluated using cheaper nutrient sources. Hence, the nutrients like nitrogen, phosphorus and potassium were replaced by a cheap and readily available fertilizer named Suphala. Thus, a modified SWM media with 220 mg/l of fertilizer Suphala resulted in a 2.59% increase in the lipid productivity. Sinha *et al*. (2017) also observed that time variant optimal light intensity and temperature trajectories, including optimum NPK fertilizer, NaCl and $NaHCO_3$ concentration has significant influence to improve its biomass and lipid productivity under minimum cultivation time and cost. Algae cost more per unit mass than other second-generation biofuel crops due to high capital and operating costs, but are claimed to yield between 10 and 100 times more fuel per unit area (Greenwell, 2009). Thus, the present study on optimization of *D. tertiolecta* growth using culture media with cheap and readily available sources of nutrients may be helpful to implement the control strategy in scale-up operation. Furthermore, the study also demonstrates that biochemical engineering-based strategies for high growth rate and lipid production of *D. tertiolecta* are strongly dependent on nutrients (nitrogen and phosphorus), salt, and temperature.

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