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EFFECT OF ORGANIC FERTILIZER ON THE GROWTH, DEVELOPMENT AND CAROTENOID PRODUCTION IN MARINE MICRO ALGAE *DUNALIELLA SALINA*

Swaminathan Detchanamurthy*[1], Deepak Kumar[1], Yashaswini Janardhanan[1], Pandiaraj Durairaj[2], Mannivannan Karunamoorthy[2]

[1*]Department of Chemical Engineering, Sri Venkateswara College of Engineering, Pennalur, Sriperumbudur, Tamilnadu, India.

[2]M/s. Seagrass Tech Pvt. Ltd, Production and R and D Facility, Akkaraivattam, Karaikal, Puducherry, India.

ABSTRACT

Dunaliella salina are exploited for various purposes of which extraction of glycerol and β carotenes are the most important. Considering the sustainability, environmental friendly nature and steady release of nutrients, organic fertilizers could be used for the cultivation of these species. The organic fertilizer 6 DRIP^{TM} was chosen for this purpose. Different concentrations of this medium (0.2-1 ml/L) were provided to grow these species and the growth rate was recorded for 16 days by measuring cell count and dry weight. The maximum dry weight (3.70 mg/L) cell counts (17726 cells/ml) and mean growth rate (0.063 divisions/day) obtained for 0.8 ml/L concentration of 6 DRIP^{TM} were similar to those obtained from Dewalne's medium (maximum dry weight 3.40 mg/L, maximum cell count 11280 cells/ml, mean growth rate 0.64 divisions/day). These samples analyzed in which the percentage of various nutrients present was recorded. β carotene level was found to be 21.89 g/100g (21.89%) of sample, which is around 21% of the dry weight. β carotene: Chlorophyll ratio was found to be 10:1 which is significant. Also, carbohydrate content was 20.18%; crude protein content was 12.20% and total lipid content was 30% of the dry biomass respectively. Thus, considering the growth rate and nutrient profile obtained for the *Dunaliella salina* grown in 6 DRIP^{TM}, this can be considered as an effective and environment friendly growth medium for the same.

KEYWORDS

Organic fertilizer, Marine micro algae, β carotene, Dewalne's medium, *Dunaliella salina* and Sea water.

Author for Correspondence:
Swaminathan Detchanamurthy,
Department of Chemical Engineering,
Sri Venkateswara College of Engineering (Autonomous),
Tamilnadu, India.

Email: dswami@svce.ac.in

INTRODUCTION

Microalgae are microscopic organisms present in both fresh water and marine systems[1]. They are being exploited for a wide range of purposes such as utilization of micro algal photosynthesis, production of various metabolites which are extensively used in the food industry, production of high value molecules[2], biofuel production, pharmaceuticals and many more. The biodiversity of microalgae is vast and *Dunaliella* are one among

them[3]. One of the best known *Dunaliella* species used for commercial purposes is the *Dunaliella salina.* Found in natural marine habitats, this alga contains high concentrations of β-carotene and also is the commercial source of glycerol[4,5]. Apart from being an antioxidant, this pigment is a non-toxic vitamin A precursor and is hence used in multivitamin formulations[6]. Since these algae are the best commercial sources of β-carotene and also have many other uses, mass cultivation has been accomplished in many countries such as USA, China, Australia, Spain, Chileetc[7,8]. Due to the increasing demand for these algae, there is a need for devising efficient and safe methods for their cultivation.

Growth mediums such as Modified Johnson's medium, Ramraj medium, F/2 medium, modified ASP (Aquatic Species Program) medium and Dewalne's Medium have been used for the cultivation of *Dunaliella salina*[9-12] so far. However, the usage of inorganic medium consumes a lot of expenditure[13]. Also, though mineral fertilizers release required nutrients quickly, their level of sustainability is less. Another factor to be considered is the heavy metal contaminants that few mineral fertilizers contain, which in the long run affect the surrounding environment (seepage into the soil etc) and also may affect the higher species (say humans) because the microalgae (like *Spirulina*) are being consumed[14]. Hence, to reduce the cost of culture medium and to provide alternative methods for growing microalgae various studies are being done currently like growing algae in industrial wastewater, usage of inexpensive nitrogen sources etc[15].

Currently few studies have been done related to usage of agricultural fertilizers for the growth of algae but extensive research has not been done in this area. This is because they are renewable, environment friendly, biodegradable sustainable and also they have the property of slow release of nutrients required for growth[16-19].

Hence, this study is aimed to use an organic fertilizer, 6 DRIP™ for the growth of *Dunaliella salina* and analyze its effect on their growth, development and pigment production.

MATERIAL AND METHODS

Microalgae source

The test algae *Dunaliella salina* was obtained from the culture collection of Seagrass Tech Private Limited, Karaikal, India.

Experimental set up

To compare the relative growth rates of *Dunaliella salina* in organic and inorganic medium the following set up (2 ml of the algae culture inoculated in 50 ml of the growth medium) was maintained (Table No.1)[20].

Inoculation and growth analysis

All the media were sterilized appropriately and conical flasks of 100 ml capacity were prepared, containing 50 ml media and 2 ml culture (inoculum). Increasing concentrations 0.2 ml/L, 0.4 ml/L, 0.6 ml/L, 0.8 ml/L, 1 ml/L of the organic fertilizer were added and appropriate concentrations of inorganic medium (control) was added to the conical flasks. The cultures were incubated at 25 ˚C in a thermo-statically controlled room with a 12:12 hr light: dark regime at a light intensity of 2500 lux as described by Jyoti Kulshreshtha, *et al*[20]. Observations were carried out every day and readings were taken once in every five days and the culture was monitored carefully in both in low carotenogenic state ("green") and high carotenogenic state ("orange"). Cultures were shaken manually twice a day to avoid clumping of cells in the bottom of the flask and accelerate growth process.

Cell count using haemocytometer

The cells were counted regularly using a haemocytometer (magnification of 10X) to check the growth of the inoculated species. This was done thrice a day and the readings obtained were noted. This data was used to calculate the cell count every day.

Dry weight analysis

For dry weight analysis, 10 ml of the samples were taken and weighed using pre weighed filter paper. These were dried in an oven at 100 ˚C and then the dry weights were measured. Formula used for calculation of growth rate using dry weights[20].

μ (divisions/day) = $3.322(\log DW_2 - \log DW_1)/t_2 - t_1$ ..... (1)

where t = time (day), DW = dry weight

Biopigment analysis

From the results obtained, the optimum concentration of organic fertilizer 6 DRIP^{TM} for the growth of *Dunaliella salina* was chosen, 100 g of the sample was subjected to testing and carotenoid, carbohydrate and crude protein, chlorophyll, lipid and fatty acid analysis was done by the Indian standard (IS) approved methods.

RESULTS AND DISCUSSION

The set of experiments were performed twice (two trials) for accurate analysis.

Figure No.1 shows the flasks containing 50 ml media and 2 ml culture with different concentrations (0.2 ml/L, 0.4 ml/L, 0.6 ml/L, 0.8 ml/L, 1 ml/L) of the organic fertilizer and appropriate concentration of inorganic medium (control) on zeroth day.

Figure No.2 represents the 16th day culture of *Dunaliella salina* (in its maturation phase) grown in 0.8 ml/L concentration of organic fertilizer.

Figure No.3: Cell counts of *Dunaliella salina* for a period of 16 days, in different concentrations of the organic fertilizer 6 DRIP^{TM}. Standard deviation represents the difference in the cell counts taken in triplicates. Commercial Dewalne's medium was used as the control, for comparison with the organic fertilizer.

Figure No.4: Dry weights (in mg/L) of *Dunaliella salina* cells for a period of 16 days, in different concentrations of the organic fertilizer 6 DRIP^{TM}. Standard deviation represents the difference in the dry weights taken in triplicates.

Figure No.5: Comparison of dry weights (eq.1) in the two trials for different concentrations of the organic fertilizer 6 DRIP^{TM}.

Dunaliella salina is capable of producing high amounts of carotenoids, especially β carotene. The growth and amount of pigment production of this species varies with different mediums and different stress conditions. After the completion of growth phase, various metabolites are produced during the maturation phase. β carotene accumulates in the inter-thylakoid spaces of the chloroplast region. The growth and bio pigment production of these vary in different types of media, under varied stress conditions. This was tested with the organic fertilizer 6 DRIP^{TM} and the observations were recorded.

The cell counts (per ml) were found to increase at a fast rate for all the concentrations provided. Five different concentrations (0.2 ml/L, 0.4 ml/L, 0.6 ml/L, 0.8 ml/L, and 1ml/L) of the organic fertilizer were tested. Dry weight analysis and cell count were carried out every day. The dry weights of the samples started increasing gradually for all concentrations of 6 DRIP^{TM} in the medium, but the rate differed for different concentrations.

From the graph (Figure No.3) it can be noticed that the cell count in the control (Dewalne's medium) increased gradually till the tenth day (growth stage) whereas, there was an acute increase from the tenth to thirteenth day and the cells reached maturation stage after the 13th day. Steep increase was noticed in the cell counts on the first day itself for 0.2 ml/L concentration of the fertilizer whereas the rate was gradual for other concentrations of the medium, showing the different rates of adaptability of the cells to grow in the mediums containing 6 DRIP^{TM}. Also, a notable increase in the amount of cells was recorded for almost all the samples (especially 0.8 ml/L) which showed that the *Dunaliella salina* adapted very well to the growth medium. In 16 days, maximum cell count was found to be 17726 cells/ml for 0.8 ml/L concentration whereas it was 11180 cells/ml for the sample grown in Dewalne's medium.

The dry weight increase was more gradual and uniform when compared with the cell count increase (Figure No.4). The dry weights increased at a gradual rate for all the samples, in particular, a steep increase in the dry weights were noticed from the 10th day, showing the steady expansion and maturation of the cells in all the sample concentrations including the control. Though there was a vast difference in the cell counts between the control and 0.8 ml/L concentration (6 DRIP^{TM}), the dry weights of both the samples were in comparable levels. At the end of the 16th day, maximum dry weight was found to be 3.63 mg/ml for the samples grown in 0.8 ml/L concentration of 6 DRIP^{TM} organic fertilizer. The initial dry weight of this sample was 1.07 mg/ml (zeroth day). The dry weight of the sample grown in Dewalne's medium

increased from1.10 mg/ml (initial concentration) to 3.43 mg/ml.

Using the dry weights obtained the growth rate (divisions/day) was calculated (Figure No.5).

μ (0.8 ml/L) = 0.0691 divisions /day

μ (Dewalne's medium) = 0.0645 divisions/day.

Figure No.6: Cell counts of *Dunaliella salina* cells for a period of 16 days, in different concentrations of the organic fertilizer 6 DRIP™. Standard deviations represent the difference in the cell counts taken in triplicates.

Figure No.7: Dry weights (in mg/L) of *Dunaliella salina* cells for a period of 16 days (obtained in the second trial), in different concentrations of the organic fertilizer 6 DRIP™. Standard deviation represents the difference in the dry weights taken in triplicates.

The same experiment was performed again and cell counts and dry weights were measured. The cell counts obtained in this trial were similar to those obtained in the first trial. The cell count increase was gradual till the tenth day and there was a steep increase on the thirteenth day for all the concentrations of organic fertilizer used. The cell counts in the control and 0.8 ml/L concentration of 6 DRIP™ crossed 11,000 cells/ml on the fifteenth day and from then; the count was almost stagnant, whereas the number of cells were not more than 10,000 cells/ml for other concentrations of the fertilizer. In few concentrations of the organic fertilizer (0.4, 0.6, 1 ml/L) the cell counts started decreasing on the 16th day, indicating the death phase, whereas the maturation phase was much more constant in 0.8 ml/L concentration of the medium. In this trial, at the end of the 16 days, maximum cell count was found to be 17700 cells/ml for 0.8 ml/L concentration whereas it was 11280 cells/ml for the sample grown in Dewalne's medium (Figure No.6). Maximum dry weight at the end of 16th day was 3.70 mg/L for the sample grown in 0.8 ml/L concentration of the organic fertilizer. The dry weight of the sample grown in Dewalne's medium increased from 1.07 mg/L (initial dry weight) to 3.40 mg/L (16th day) (Figure No.7).

Using the dry weights obtained the growth rate (divisions/day) was calculated (Figure No.5).

μ (0.8 ml/L) = 0.05760 divisions /day

μ (Dewalne's medium) = 0.0641divisions/day.

From both the trails, the mean growth rate value was found to be 0.06335 divisions/day for 0.8 ml/L concentration of the organic fertilizer used which is comparable to the mean growth rate value obtained in Dewalne's medium.

In both the trials, the dry weight and cell counts obtained for 0.8 ml/L concentration of 6 DRIP™ (maximum dry weight 3.70 mg/L, maximum cell count 17726 cells/ml approx.) were in comparable levels to that obtained from Dewalne's medium (inorganic medium) (maximum dry weight 3.40 mg/L, maximum cell count 11280 cells/ml). Though there were major differences in the number of cells between control and 0.8 ml/L concentration, the dry weights were similar which indicates that the cells multiplied rapidly during the growth phase but the expansion (cell size) and maturation was similar to that of those cells in Dewalne's medium. For other concentrations of this fertilizer (say 0.6 ml/L and 1ml/L) the cell death started occurring in the 16th day which could be due to deficit (in Trial No.1) and inundating (Trial No.2) concentration of nutrients.

So, 0.8 ml/L concentration was considered as optimum and the samples were out sourced to SMS LABS SERVICES PRIVATE LIMITED, Tamil Nadu 600124, India and the result of analysis were obtained as follows: β carotene level was found to be 21.89 g/100g (21.89%) of sample, which is around 21% of the dry weight. β carotene: Chlorophyll ratio was found to be 10:1 which is significant. Also, carbohydrate content was 20.18% of the dry weight; crude protein content was 12.20% of the dry weight and total lipid content was 30% of the dry biomass. Cells experience nitrogen and phosphorous starvation as these levels are supplied less from the organic fertilizer 6 DRIP™ but this starvation subsequently lead to more accumulation carbohydrate and lipids when compared to the accumulation of proteins. Overall, these levels of bio pigment, lipid, carbohydrate and protein were similar to those obtained from the species grown in Dewalne's medium.

Also, analysis of the medium composition of this organic medium 6 DRIP™ shows that this medium contains various nutrients (needed for growth and

maturation) in required quantities. Organic carbon (basically essential for photosynthesis) is present in the form of bicarbonate that is required by the cells for high light and ion tolerance. Nitrogen is present in the form of nitrate (4.047 g/L) which is used for growth and protein synthesis. Phosphorus (phosphate being an integral part of essential molecules such as ATP (Adenosine Triphosphate), the energy carrier in cells, also the backbone in DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid)) and sulphur (important constituents of amino acids) are provided as phosphate (0.073 g/L) and sulphate (19.088 g/L). Other constituents required for growth such as potassium (6.385 g/L), calcium (0.319 g/L), magnesium (0.192g/L), chloride (11.093 g/L) and sodium (8.624 g/L) are also present in the medium.

Though zinc, a toxic metal, is present in the medium, it is present in very low concentrations (0.016 g/L) and hence doesn't affect the growth of the cells in a negative manner. Mg^{2+}:Ca^{2+} ratio is found to be 0.60 whereas Cl^-:SO_4^{2-} ratio is 0.60. Iron (an important trace metal for normal growth and functioning of photosynthesis and respiration) is present in low (yet optimum) concentrations for growth (0.466 g/L). Since these species have a wide range of pH tolerance (1-11), a pH of 10.56 provided by the medium serves to ideal for growth. Copper, cobalt and molybdenum are below detection levels in the medium, but these are present in the sea water and hence are utilized by the organisms for their growth.

Thus, this medium serves to be optimum for the growth and maturation of *Dunaliella salina.*

Table No.1: Comparison of organic and inorganic growth medium

S.No	Nutrients	Concentration in Dewalne's medium	Concentration in 6 DRIP™
1	Nitrogen as NO_3	99 g/L	4.047 g/L
2	Phosphorus as PO_4	20.53 g/L	0.073 g/L
3	Potassium as K	BDL	6.385 g/L
4	Chloride as Cl	0.81228 g/L	11.093 g/L
5	Sulphate as SO_4	0.07695 g/L	9.098 g/L
6	Calcium as Ca	BDL(Below Detection Limit)	0.319 g/L
7	Magnesium as Mg	BDL	0.192 g/L
8	Sodium as Na	36.355 g/L	8.624 g/L
9	Copper as Cu	0.05092 g/L	BDL
10	Zinc as Zn	0.01006 g/L	0.016 g/L
11	Molybdenum as Mo	0.04891 g/L	BDL
12	Cobalt as Co	0.4953 g/L	BDL
13	Ferric as Fe	0.27 g/L	0.466 g/L
14	Manganese as Mn	0.111 g/L	BDL

Table No.2: Sample analysis of 0.8 ml/L concentration

S.No	Parameter	DW Percentage level
1	β carotene	21%
2	Carbohydrate	20.18%
3	Crude protein	12.20%
4	Total lipid	30%

Figure No.1: Zeroth Day

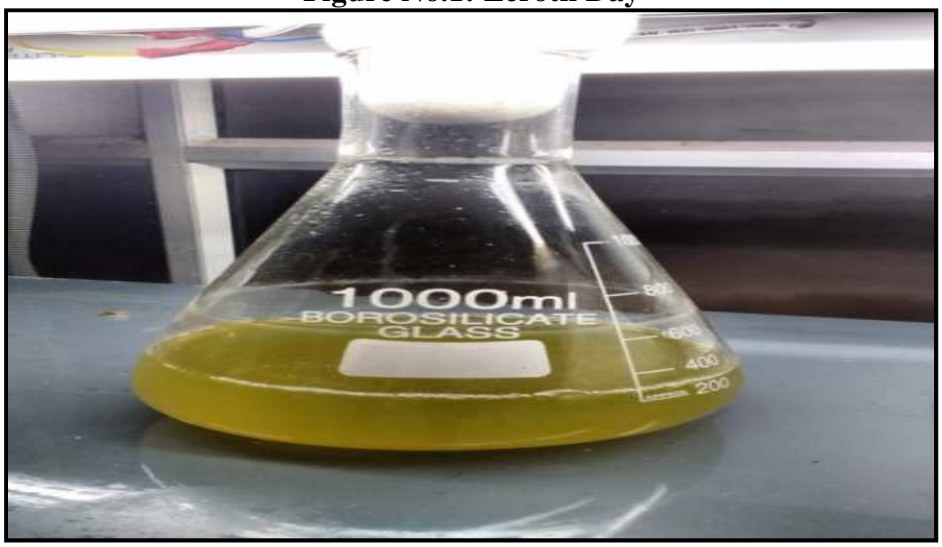

Figure No.2: 16th Day

Trial 1

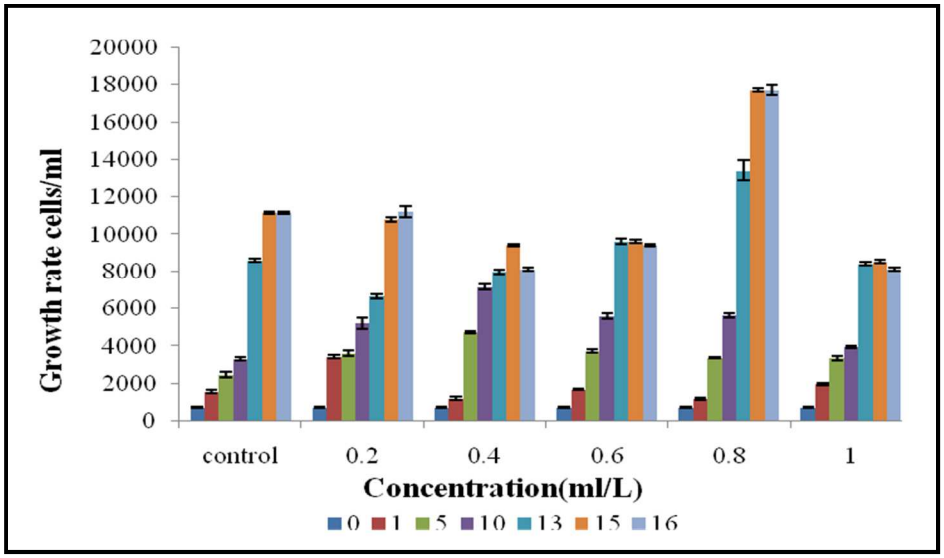

Figure No.3: Cell counts of *Dunaliella salina*

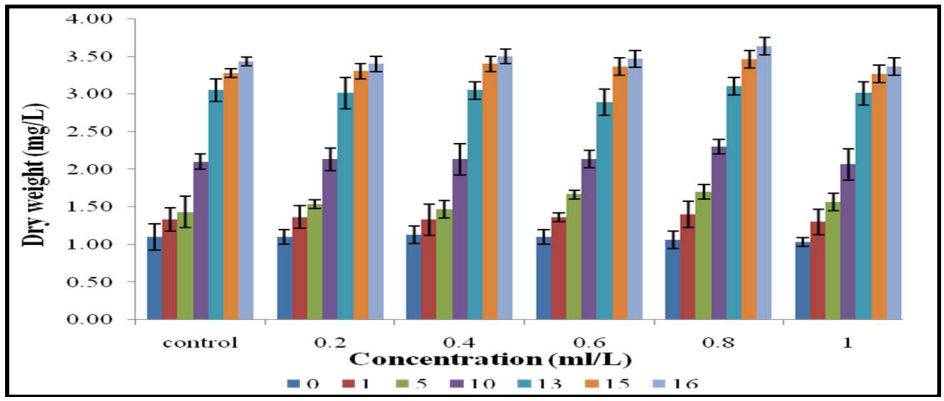

Figure No.4: Dry weights (in mg/L) of *Dunaliella salina cells*

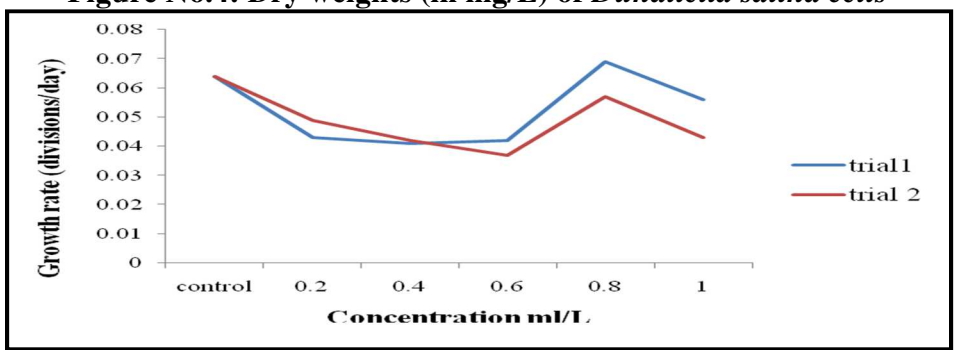

Figure No.5: Comparison of dry weights (eq.1) in the two trials for different concentrations of the organic fertilizer 6 DRIP™

Trial 2

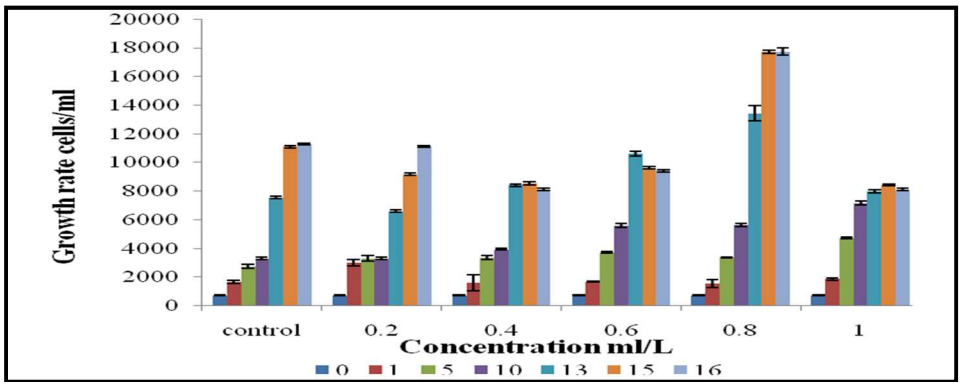

Figure No.6: Cell counts of *Dunaliella salina*

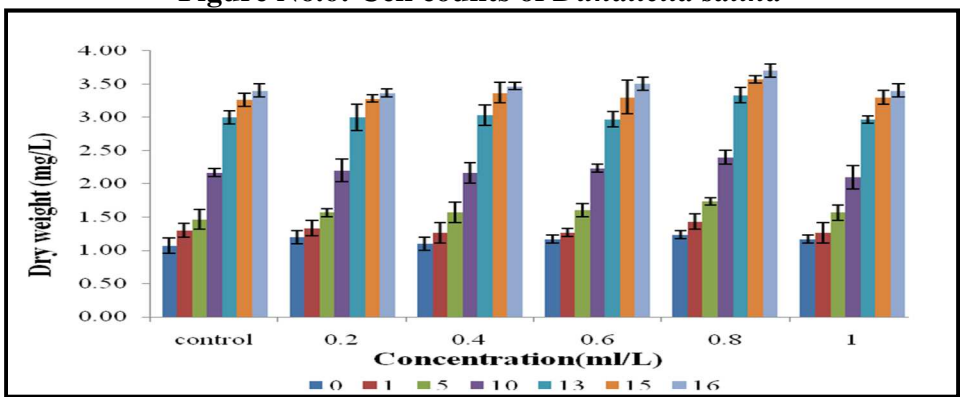

Figure No.7: Dry weights (in mg/L) of *Dunaliella salina* cells

CONCLUSION

This study examined the various effects (positive and negative) of using the organic fertilizer 6 DRIP ™ on the growth and expansion of *Dunaliella salina*. Being highly demanded for commercial purposes, increased production levels of the microalgae *Dunaliella salina* has interested many countries in the world. While cultivating these organisms on a large scale, managing the expenditure as well as reducing the environmental damage becomes necessary to enhance the quality and retain the sustainability of this process. On this basis, the organic fertilizer 6 DRIP ™ was used and the cells were grown in different concentrations of this medium. The growth rate and metabolite production of cells in 0.8 ml/L concentration of the medium was comparable to that of those grown in inorganic medium. High levels of β carotene (21.89% DW), carbohydrate (20.18% DW), protein (12.20% DW) and lipids (30% DW) were produced by the cells grown in the organic fertilizer due to nitrogen and phosphate starvation. Also, significant β carotene: Chlorophyll ratio (10:1) was obtained. On analyzing the nutrient profile of the medium, it can be noticed that the level of nutrients present is similar to those levels in the commercially available Dewalne's medium with the exception of few (nitrogen, phosphate and potassium). Also, the cells are able to adapt and grow effectively in the nutrient conditions provided. Not only the growth, but also the metabolite production is found to be effective in this medium. Hence, this research provides a start to the idea of using organic fertilizers for the growth of *Dunaliella salina* and further extensive research is required to optimize the growth conditions and check the effect of various factors on the growth and biopigment production of the micro algae *Dunaliella salina*.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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