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Effect of shaking, incubation temperature, salinity and media composition on growth traits of green microalgae *Chlorococcum* sp.

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Abasract

Biofuel derived from microalgae are considered as a highly promising next-generation fuel. *Chlorococcum* sp. (green microalgae) shows potential for the successful biodiesel production, where biodiesel is produced from neutral lipid content of the algae. In the present study, effect of shaking, incubation temperature, salinity and different media composition on various physiological and biochemical traits like chlorophyll, protein, carbohydrate, biomass and lipid contents of green microalgae *Chlorococcum* sp. were assessed. Most of the traits were found higher in shaking condition except the lipid accumulation. When isolated *Chlorococcum* sp. is grown at 25 ^{0}C, the higher yields of lipid and chlorophyll contents were observed. The effect of various concentrations of NaCl on the isolated algal species of *Chlorococcum* spp. has showed increased biomass yield at 0.2mM NaCl concentration as compared to control. Initial increase of NaCl concentration from 0.0-0.2 mM decreased the lipid accumulation. The effect of culture media composition showed that BG-11 and BBM are the best suited media for the growth of this species. The present study signifies that the growth of microalgae not only depends on the temperature, light and nutrient availability, but is also highly affected by the salinity and culture media composition.

Keywords: BG-11, Biomass, Carbohydrate, Chlorophyll, *Chlorococcum* spp., NaCl.

Introduction

The world has been confronted in recent decades with an energy crisis, associated with irreversible depletion of traditional sources of fossil fuels; their use as major form of energy is indeed unsustainable, further the accumulation of greenhouse gases in the atmosphere brings about global warming. Compared with other forms of renewable energy (e.g. wind, tidal and solar), biofuels allow energy to be chemically stored, and can also be used in existing engines and transportation infrastructures after blending to various degrees with petroleum diesel (Singh and Gu 2010). The biodiesel is receiving widespread attention with respect to its non-toxicity, biodegradability and as a renewable source of energy. Production of algae-based liquid fuels is being intensively investigated by nearly every major oil company (Mascarelli 2009; McCoy 2009; Voith 2009; LeBlanc 2008) as a potential replacement for petroleum. Algal crude oil and biomass are potentially important renewable feedstocks for the future chemical industry (Gavrilescu and Chisti 2005). Microalga is a photosynthetic microorganism that is able to use the solar energy to combine water with carbon dioxide to create biomass. Microalgae culture is similar to any eukaryote cell culture. It grows in three main phases: lag, exponential and stationary. They grow at an exceptional fast rate: 100 times faster than terrestrial plants and they can double their biomass in less than one day (Tredici 2010). Apart from that, some microalgae strains are able to accumulate large quantity of lipid inside their cells, which can be converted to biodiesel (Chisti 2007). Microalgae can produce 30 times more oil than terrestrial oilseed crops for a given surface area (Sheehan *et al.* 1998). The main biodiesel producing microalgae are *Botryococcus braunii*, *Chlorella* spp., *Chlorococcum* spp. etc. (Hirata *et al.* 1996; Murakami and Ikenouchi 1997).

In view of changing energy scenario for renewable energy sources *Chlorococcum* spp. is one of the best known species for its biodiesel production capacity. Large-scale production of *Chlorococcum* biomass depends on many factors, the most important of which are nutrient availability, salinity, temperature and light. These factors influence the growth of *Chlorococcum* and the composition of the biomass produced by causing changes in metabolism. The biomass of algal species mainly comprises of protein, carbohydrate, and lipids (Spolaore *et al.* 2006). Number of media compositions for the cultivation of microalgae has been proposed. The elements required for the growth of green algae are N, P, K, Mg, Ca, S, Fe, Cu, Mn, and Zn (Oh-Hama and Miyachi 1988). The composition of intracellular lipid of

microalgae was reported to change in response to environmental salinity. Increase of NaCl concentration from 0.4M to 4M increased saturated and monounsaturated fatty acids in *Dunaliella* cells isolated from an Antractic hypersaline lake (Xu and Berdall 1997), while polyunsaturated fatty acid decreased. The selection of best medium especially when growing cryptogams like algae is prerequisite for any research endeavors. Different nutrient composition can influence the growth parameters of algae; hence, it can be exploited for optimization of culture conditions. Since most of our laboratory studies are made with pure cultures, one must be able to sterilize a culture medium and maintain it in a sterile condition.

In the present study impact of NaCl concentration, shaking speed, incubation temperature and different media compositions like BG-11, Bold's Basal medium (BBM), Chu#10, Half-Strength (HS) Chu#10, Allen on the growth traits of an algae *Chlorococcum* spp. has been evaluated.

Materials and Methods

The experimental organism green microalga *Chlorococcum* sp. was isolated from water sample collected from a freshwater pond from village Ladwi, Hisar (Haryana). The water sample was having pH 7.5. Pure culture of *Chlorococcum* spp. was obtained by repeated streaking and plating at pH 7.0±1 using standard isolation and culturing techniques in BG-11 medium. The algal sample was cultured on BG-11 medium and maintained on the same medium by regular subculturing in every two weeks. Cultures were maintained at a light intensity of 3000 lux using cool fluorescent tubes at 25±1^{o}C in algae culture room. The identification of algal cultures was done by observing under compound microscope up to genera level with the help of Professor B. B. Chaugule, Department of Botany, University of Pune, Pune, India.

Effect of shaking speed was evaluated in BG-11 medium at 30^{o}C and 120 rpm and control was also run parallel under static condition. The culture flasks were maintained under similar environmental condition for 14 days by running the experiments parallel. To determine the effect of incubation temperature, cultures were grown at different temperatures of 23^{o}C, 25^{o}C, 27^{o}C, 30^{o}C and 35^{o}C at 120 rpm for 11 days.

Further to study the impact of NaCl, the algal species was grown in BG-11 medium modified with varying salt concentrations (0.2 mM to 1.0 mM). Appropriate dosing was made from 1000 mM NaCl stock solution. To study the effect of salinity on *Chlorococcum* spp. the experiments were carried out in 250 ml Erlenmeyer flasks each containing 100 ml of BG-11 medium incubated at 25^{o}C in an orbital shaker set to120 rpm in BOD incubator cum shaker for 15 days and control culture in BG-11 media was also run parallel. The medium and flasks were sterilized in an autoclave for 20 min at 121^{o}C in order to prevent any contamination. The samples were drawn on 15th day and were subjected to analysis for various physiological and biochemical parameters. All the experiments were carried out at least in duplicate.

In order to find out the best culture medium, cultures were subjected to five media of different chemical compositions and pH, such as BG-11, Bold Basal Medium (BBM), Chu#10, HS Chu#10, Allen media. All the media compositions used in our experimental study is described in Table-1.

Table-1: Composition of different medias used in the study.

Ingredients (g/l)	Different Media				
	BG-11	**BBM**	**Chu#10**	**HS-Chu#10**	**Allen**
$NaNO_3$	1.5	0.25	-	-	-
K_2HPO_4	0.04	0.075	0.5	0.0025	-
$MgSO_4•7H_2O$	0.075	0.075	2.5	0.0125	0.247
$CaCl_2•2H_2O$	0.036	0.025	-	-	-
Citric acid	0.006	-	-	-	-
Ferric ammonium citrate	0.006	-	-	-	-
EDTA (disodium salt)	0.001	-	-	-	-
Na_2CO_3	0.02	-	2.0	0.010	-
KH_2PO_4	-	0.175	-	--	0.272
NaCl	-	0.025	-	-	-
$Ca(NO_3)_2$	-	-	4.0	0.02	-
Na_2SiO_3	-	-	2.5	0.0125	-
$FeCl_3$	-		0.08	0.0004	-
$(NH_4)_2SO_4$	-	-	-	-	1.32
$CaCl_2$	-	-	-	-	0.055

Trace metals solution having composition (H_3BO_3 2.86; $MnCl_2$•$4H_2O$ 1.81; $ZnSO_4$•$7H_2O$ 0.222; $NaMoO_4$•$2H_2O$ 0.39; $CuSO_4$•$5H_2O$ 0.079; Co $(NO_3)_2$•$6H_2O$ 0.0494)	1ml	-	-	-	-
Alkaline EDTA solution having composition (EDTA 0.05; KOH 0.031)	-	1ml	-	-	-
Acidified Iron solution having composition ($FeSO_4$ •$7H_2O$ 0.00498 ; H_2SO_4 1-2 drops)	-	1ml	-	-	-
Boron solution having composition (H_3BO_3 0.01142)	-	1ml	-	-	-
Trace metals solution having composition ($ZnSO_4$ •$7H_2O$ 0.00882; $MnCl_2$•$4H_2O$ 0.00144; MoO_3 0.00071; $CuSO_4$•$5H_2O$ 0.00157; $Co(NO_3)_2$•$6H_2O$ 0.00049)	-	1ml	-	-	-
Trace metals solution having composition (H_3BO_3 0.00248 ; $MnSO_4$•H_2O 0.00147; $ZnSO_4$•$7H_2O$ 0.00023; $CuSO_4$•$5H_2O$ 0.0001;$(NH_4)_6Mo_7O_{24}$•$4H_2O$ 0.00007; $Co(NO_3)_2$•$6H_2O$ 0.00014)	-	-	-	1ml	-
Vitamins solution having composition (Thiamine•HCl (Vitamin B_1) 0.050; Biotin (Vitamin H) 2.5; Cyanocobalamin (Vitamin B_{12}) 2.5)	-	-	-	1ml	-
Trace metals solution having composition (Fe-Na- EDTA•$3H_2O$ 0.03016; H_3BO_3 0.00286; $MnCl_2$•$4H_2O$ 0.00179; $ZnSO_4$•$7H_2O$ 0.00022; $CuSO_4$•$5H_2O$ 0.000079; $(NH_4)_6Mo_7O_{24}$•$4H_2O$ 0.00013; NH_4NO_3 0.000023)	-	-	-	-	1ml

All the growth experiments were carried in 250 ml Erlenmeyer flasks each containing 100 ml of different media incubated at 25±2^{o}C in culture room. The medium and flasks were sterilized in an autoclave for 20 min at 121^{o}C in order to prevent any contamination. One ml ten days fresh inoculum was used for all the experiments, keeping care that the cultures do not get too old and reach late stationary phase as depletion of nutrients and accumulation of waste products causes deterioration and damage to the cultures. The growth of algal isolate was measured in terms of various physiological and biochemical parameters like biomass, chlorophyll, protein, carbohydrate and lipid contents. Dry cell biomass was measured as cell density (dry cell weight g/l) at OD_{625} of 11 days old culture at dilutions ranging from 0.2 to 1.0. The dry biomass was calculated by using the regression equation:

$Y = 1.014x + 0.249$; $R^2 = 0.965$

where Y (g/l) is cell density and x is OD_{625}.

Chlorophyll content of the algae was estimated spectrophotometrically at 650 and 665nm by hot extraction method of Tandeau de Marsac and Houmard (1988). The chlorophyll content was calculated using the following formula and was expressed as µg/ml:

Chlorophyll (µg /ml) $=2.55 \times 10^{-2} OD_{650} + 0.4 \times 10^{-2} OD_{665} \times 10^3$

Protein content was estimated at 660 nm by the method of Lowry and coworkers (Lowry *et al.* 1951). Carbohydrate was determined by anthrone reagent method (Dubois *et al.* 1956).

Total lipids were extracted by mixing methanol-chloroform (2:1.5 v/v) with the algal samples using slightly modified version of Bligh and Dyer's method (Bligh and Dyer 1959).

Results and Discussion

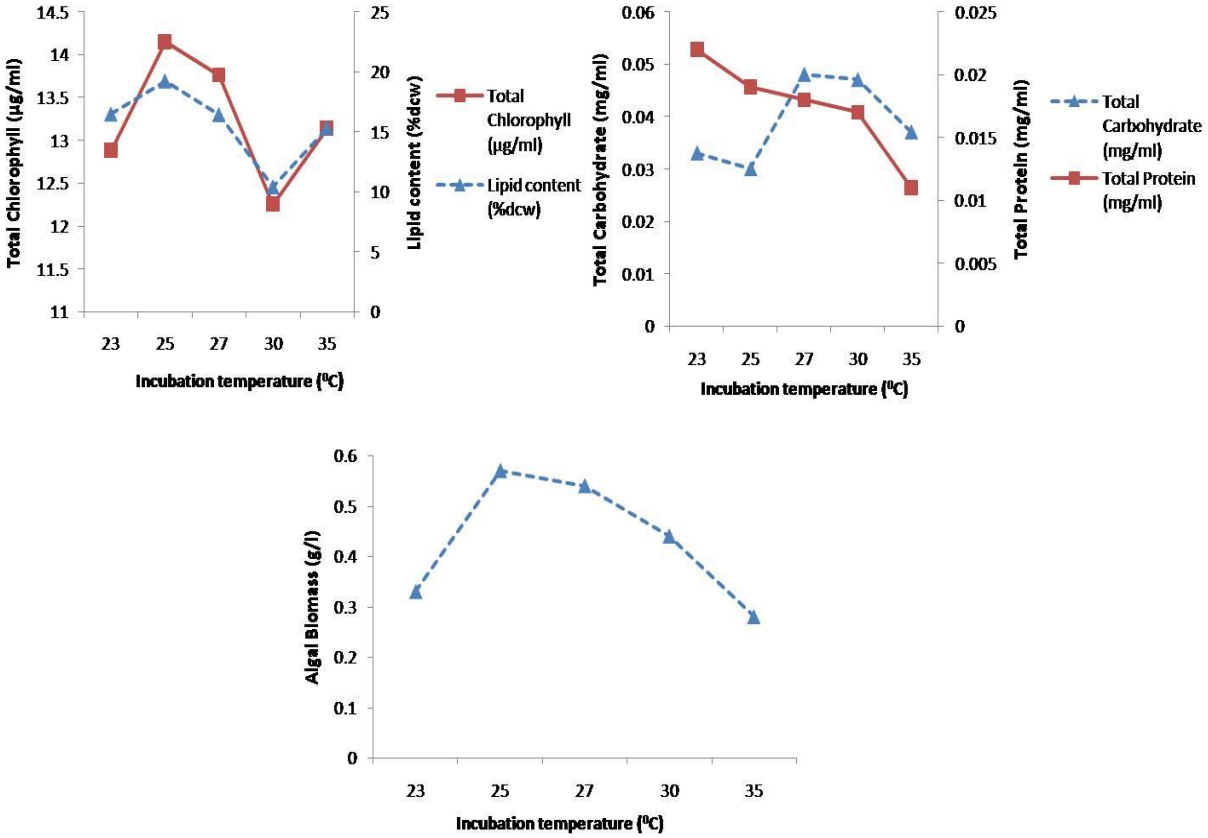
Static cultures were found to be having higher values of the lipid accumulation (18.35 %) in comparison to shaking conditions (13.89 %). However, chlorophyll (10.65 µg/ml), protein (0.021 mg/ml) and carbohydrate (0.047 mg/ml) contents were higher in shaking condition (Table- 2).

Table-2: Effect of static and shaking conditions on various growth parameters of green microalgae *Chlorococcum* spp.

Culture conditions	Algal Biomass (OD) (g/l)	Lipid Content (%dcw)	Total Chlorophyll (µg/ml)	Total Carbohydrate (mg/ml)	Total Protein (mg/ml)
Static .	0.34	18.35	7.18	0.029	0.012
Shaking .	0.35	13.89	10.65	0.047	0.021

Lipid content accumulated (19.23%dcw), chlorophyll content (14.15 µg/ml) and algal biomass (0.57 g/l) yield were found highest when *Chlorococcum* sp. was grown at incubation temperature of 25^{o}C at 120 rpm for 11 days (Fig. 1). The results indicated that lipid accumulation and chlorophyll contents were favoured at 25^{o}C.

Fig. 1: Effect of different incubation temperatures on various growth traits of *Chlorococcum* spp. on 15th day of growth

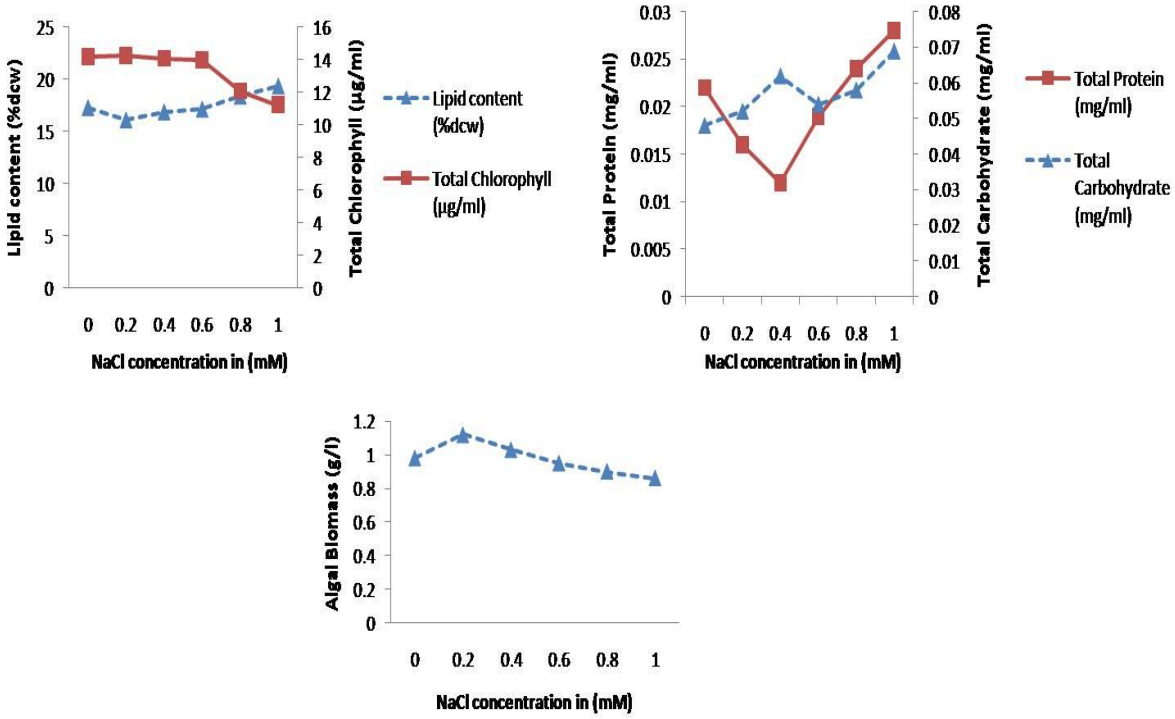


Impact of salinity on different physiological and biochemical traits of *Chlorococcum* spp. is shown in Fig. 2. The algal biomass yield was highest at 0.2mM NaCl concentration as compared to control and then it subsequently decreased with increase in NaCl concentration. The initial increase of NaCl concentration from 0.0-0.2 mM decreased the lipid accumulation from 17.26% to 16.09 % dcw. The lipid content increased gradually when the NaCl concentration is increased from 0.2-1.0 mM. The increase in lipid content at higher NaCl concentration may be due to of accumulation of lipid content under stress conditions. These results are in accordance with the finding of Takagi and his coworkers (Takagi *et al.* 2006). They found the similar results in *Dunaliella* cells. Total chlorophyll contents decreased as the salt concentration is increased from 0.2 to 1.0mM when compared to control for the culture studied. According to Moradi and Ismail (2007), reduced chlorophyll contents at higher salinities are due to decrease in photosynthetic rate because of salt osmotic and toxic ionic stress. Many previous studies reported that the cultivation under higher saline concentrations had lowered chlorophyll and protein contents (Vonshak *et al.* 1996). It has also been reported that chlorophyll is primary target to salt toxicity limiting net assimilation rate, resulting reduced photosynthesis and reduced growth (Rai 1990; Rai and Abraham 1993). Carbohydrate content increased in all the concentrations of NaCl as compared to control for the culture studied. Many previous studies reported that carbohydrates synthesis was stimulated by stress conditions (Warr *et al.* 1985; Tomaselli *et al.* 1987). Algae exhibited decline in total protein content at NaCl concentrations of 0.2 and 0.4mM and thereafter NaCl concentration of ≥0.6mM, the quantity of total protein content increased. Here results of total protein content are in accordance with the finding of Vonshak *et al.* (1996) and they reported that the cultivation with higher saline concentrations had lowered chlorophyll and protein contents.

Table-3: Effect of different media compositions on various growth parameters of green microalgae *Chlorococcum* spp.

Different Media	Algal Biomass (OD) (g/l)	Lipid Content (%dcw)	Total Chlorophyll (µg/ml)	Total Carbohydrate (mg/ml)	Total Protein (mg/ml)
BG-11	0.95	13.12	13.4	0.029	0.025
Bold's Basal .	0.31	19.25	13.87	0.036	0.016
HS Chu#10	0.49	13.24	9.79	0.055	0.022
Chu#10	0.44	14.22	6.71	0.065	0.019
Allen	0.28	18.36	6.22	0.041	0.016

Fig. 2: Effect of NaCl on various growth traits of *Chlorococcum* spp. on 15th day of growth



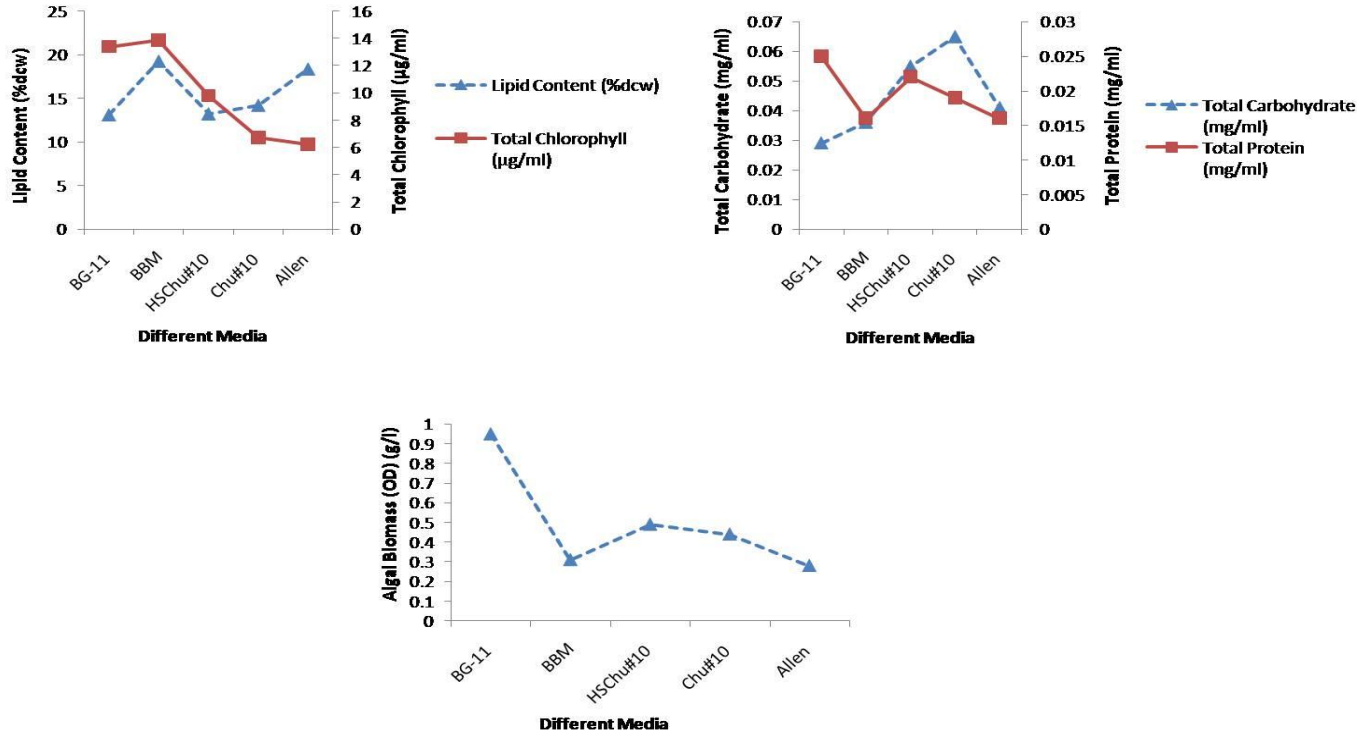


Fig. 3: Effect of different media compositions on various growth parameters of *Chlorococcum* spp.

The effect of different media on various growth parameters is shown in Table-3 and Fig. 3. The results indicated that, the algal biomass yield was highest (0.95 g/l) in BG-11 medium and chlorophyll content was slightly higher (13.87 µg/ml) in BBM than BG-11 medium (13.40 µg/ml). This implied that with increase in algal biomass there is also an increase in chlorophyll content of algal strain. The findings of algal biomass yield are in accordance with the results of Eroglu and Melis (2010) obtained in algal species *Botryococcus braunii*. The total chlorophyll content of cells in BBM was highest reflecting on the enhanced capacity of the medium to maintain heterotrophic cultures. Lipid content was maximum in BBM (19.25 % dcw) followed by Allen medium (18.36 % dcw). The higher value of lipid content might be because of lower level of nitrates in the media as similar results of higher lipid contents in green algae *Chlorella vulgaris* at lower $NaNO_3$ and KNO_3 were reported by Tornabene *et al.* 1983. Carbohydrate comes out to be maximum in Chu#10 (0.065 mg/ml) followed by HS Chu#10 medium (0.055 mg/ml), whereas BG-11 supports the best protein content (0.025 mg/ml) followed equally by BBM and Allen media (0.16 mg/ml). The results of maximum protein content are in accordance with the findings of Shilpkar *et al.* 2010.

Conclusion

The present study signifies that the growth of microalgae not only depends on the temperature, light and nutrient availability, but is also highly affected by the salinity and culture media composition. Higher lipid, chlorophyll and biomass content was observed when culture of *Chlorococcum* spp. is grown at 25 ^{0}C. The effect of various concentrations of NaCl on the isolated algal species of *Chlorococcum* spp. showed, increased biomass yield at 0.2mM NaCl concentration as compared to control and then it subsequently decreases with increase in NaCl concentration. Initial increase of NaCl concentration from 0.0-0.2 mM decreased the lipid accumulation from 17.26 to 16.09 % dcw. The effect of culture media composition showed that BG-11 and BBM are the best suited media for the growth of this species.

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