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Effect of Different Culture Media on Cell Concentrations of *Chlorella Pyrenoidosa* under Photoautotrophic Conditions

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Abstract

The influence of light intensity and media on the growth of the freshwater microalga *Chlorella pyrenoidosa* has been studied. Four media each with 20, 35 and 55 µmol photons $m^{-2}s^{-1}$ light intensities resulting in twelve different combinations were studied. As light intensity increased from 20 µmol photons $m^{-2}s^{-1}$ to 55 µmol photons $m^{-2}s^{-1}$, there was a significant increase in cell concentration in all the media. Of the different growth patterns observed, maximum cell concentration of 6.85 x 10^6 cells per ml was obtained in BG11 medium at 55 µmol photons $m^{-2}s^{-1}$ corresponding to a specific growth rate (µ) of 0.327d^{-1} with a least doubling time (t_d) of 50.86h. It was observed from this study that as light intensity increased from 20 µmol photons $m^{-2}s^{-1}$ to 55 µmol photons $m^{-2}s^{-1}$ media had no significant effect on the growth of alga.

Key words: Cell count, Light intensity, Medium composition, Specific growth rate, Doubling time

Nomenclature

µ: specific growth rate (d^{-1})
MBM: Mod. Basal Medium
RM: Rudic's Medium
CM: Chlorella Medium
BGM: BG11 Medium
t: culture time (days)
t_d: doubling time (h)
ANOVA: Analysis of Variance
F_{cal}: Calculated F value using ANOVA
F_{Crit} : Critical F value in F-test
SD: Standard deviation

INTRODUCTION

The broad utilities of microalgae include capability of producing multiple products, ranging from energy, chemicals and materials to applications in carbon sequestration and wastewater remediation. Microalgae are cultivated commercially for human nutritional products around the world in several small- to- medium scale production systems, thus producing several hundreds of tonnes of biomass annually. The main photosynthetic algae generally cultivated for various nutritional products are *Spirulina, Chlorella, Dunaliella* and *Haematococcus*. The total world production of dry algal biomass for these four algae is estimated to be about 10,000 tonnes per year. About half of this production takes place in China, with most of the rest in Japan, Taiwan, U.S.A, Australia and India, and few other countries [1].

Currently cultivation of *Chlorella* is most frequently carried out phototrophically in open ponds. However, some restrictions such as light intensity, period of light, temperature, etc. severely limit its development [2, 3].Autotrophic growth of *chlorella* does provide several advantages: (a) microalgae can harvest the radiant energy from the sun into valuable products at the expense of inexpensive natural resources like CO_2 and H_2O, which contributes to global CO_2 reduction [4]; (b) microalgae can bloom at places where salty water, excessive sun exposure, and lack of vital nutrients inhibits other crops to grow [5, 6].

In spite of the advantages autotrophic growth has a drawback of producing lower biomass when compared with heterotrophic conditions .However in another study, the lipid content in autotrophic conditions were higher when compared with heterotrophic growth conditions[7]. Pratt and Johnson compared protein and lipid contents of *C. vulgaris* and *C. pyrenoidosa* and revealed that *C. vulgaris* yields about 14 per cent less protein and nearly 30 per cent less lipid than *C. pyrenoidosa* [8]. We hypothesize that lipid productivity can be increased if biomass is increased in autotrophic growth conditions of *C. pyrenoidosa* which can then be used as a potential lipid source for biodiesel production.

Little information is available relating to the autotrophic growth of *C. pyrenoidosa* [9]. It is known that of various parameters used for increasing the biomass productivity, nutrient media and light intensities play a major role [5]. Till date there are no reports associated with the comparative studies of different culture media and light intensities on growth of *C. pyrenoidosa* for increasing biomass yield under autotrophic conditions. With this objective, the present study was carried out to study the effect of cell concentrations in photoautotrophic growth conditions of *C. pyrenoidosa* in different culture media & light intensities.

MATERIALS and METHODS

Algal strain and Inoculum preparation

Chlorella pyrenoidosa (NCIM NO: 2738) was obtained from the National Collection of Industrial Micro-organisms (NCIM), Pune, India. Stock culture of *C. pyrenoidosa* was grown photoautotrophically in - Chlorella medium (CM) (Table 1). The pH of the medium was adjusted to 7.5 and the temperature was maintained at 33 °C under illumination (16:8 day-night cycle) at 20 µmol photons m^{-2} s^{-1} in 1-L flask. A constant air flow rate of 1 L min^{-1} was maintained. For the preparation of inoculum, stock cultures grown in four different media viz., Rudic's Medium (RM) [10], BG11medium (BGM) [11], Modified Basal medium (MBM) [12] and CM [13] were centrifuged for two minutes at 2000 rpm and the pellet was transferred aseptically into four 250 ml Erlenmeyer flasks containing 90 ml of respective media, under above mentioned conditions for 5 days. Light intensity was measured with a LUX meter (LM-52-780) and illumination was provided by standard cool white fluorescent lamps.

Exponential phase of *C. pyrenoidosa* starts from 5th day under 20 µmol photons m^{-2} s^{-1}. Cell count was normalized to 0.6 x10^6 cells per ml in all the media on 5th day. 10ml of this 5-day old culture (0.6 x10^6 cells per ml) was inoculated into 90 ml sterilized fresh media in 250 ml Erlenmeyer flasks. On the day of inoculation the initial cell count was 0.06 x10^6 cells per ml in all the samples .The flasks were incubated for 14 days at 33°C under three light intensities of 20, 35, 55 µmol photons $m^{-2}s^{-1}$ in four different media (CM, BGM, RM & MBM) .

The cell concentration was determined by counting triplicate samples in a Neubauer haemocytometer. The mean and standard deviation of the three samples were assessed. The specific growth rate (µ) of the culture was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = (\ln X_2 - \ln X_1)/\Delta t$, where X_2 is the final cell concentration, X_1 is the initial cell concentration and Δt is the time required for the increase in concentration from X_1 to X_2. The doubling time of *C .pyrenoidosa* was also calculated using the equation $t_d = (24 * \ln 2)/\mu$. Statistical tests were performed by comparing the cell concentrations in different light intensities using Analysis of Variance (ANOVA) for finding out the effect of media on the growth of the C*hlorella* with varying light intensities.

RESULTS

The effects of 12 different combinations on the growth of *Chlorella pyrenoidosa* were simultaneously investigated for 14 days of cultivation period. There were significant differences on the growth of cells beginning from 7th day of cultivation in different culture media at a light intensity of 20 µmol photons $m^{-2}s^{-1}$ (Yadavalli Fig 1). Maximum cell concentration of 3.191 x 10^6 cells per ml was achieved in RM on the 13th day of cultivation.

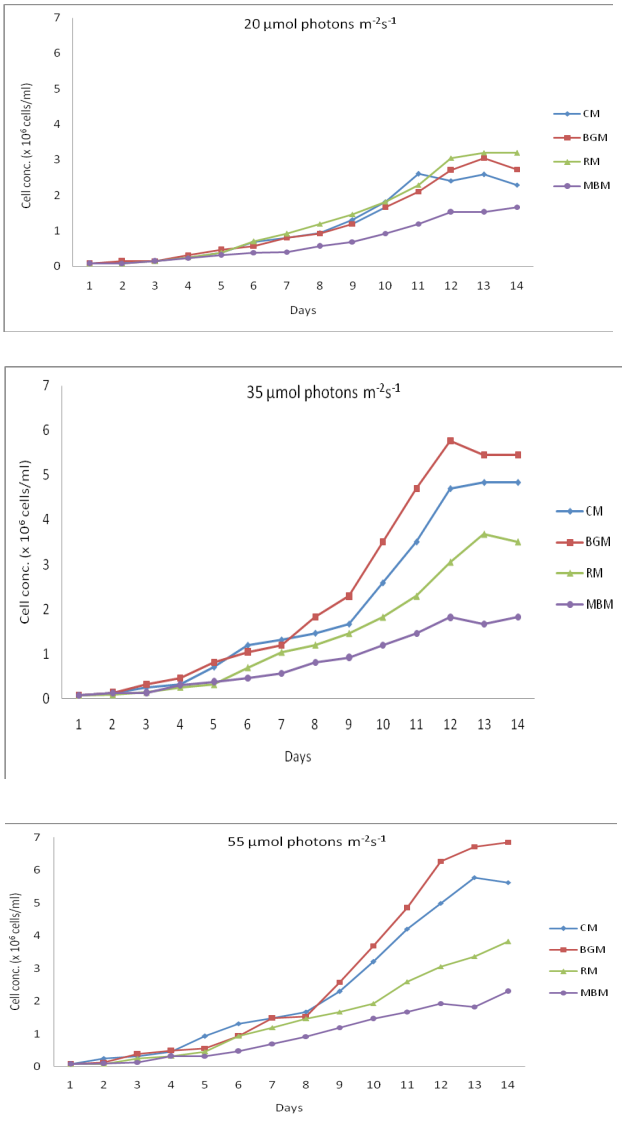
Table1. Composition of different culture media used for the growth of *C. pyrenoidosa*

Constituent (g/l)	CM	BGM	RM*	MBM
KNO_3	2		0.3	1.25
$NaNO_3$		1.5		
K_2HPO_4	0.1	0.04	0.08	1.25
KH_2PO_4			0.02	
$MgSO_4 \cdot 7H_2O$	0.1	0.075	0.01	1
$CaCl_2 \cdot 2H_2O$		0.036	0.058	0.11
Citric acid		0.006		
$NaCO_3$		0.02		
$CaCl_2 \cdot H_2O$	0.05			
NaCl			0.02	
H_3BO_3	0.00286	0.00286	0.003	0.0011
$MnCl_2 \cdot 4H_2O$	0.00181	0.00181		0.00015
$ZnSO_4 \cdot 7H_2O$	0.00022	0.00022	0.001	0.0009
$NaMoO_4 \cdot 2H_2O$	0.0004	0.00039		
$CuSO_4 \cdot 5H_2O$	0.00008	0.00008	0.0008	0.00016
$Co(NO_3)_2 \cdot 6H_2O$		0.00005	0.0026	0.00005
$(NH_4)_6Mo_7O_{24}$ $4H_2O$		0.003	0.0001	
MnSO4			.015	
$FeSO_4 \cdot 7H_2O$	0.00557			0.0005
Na_2EDTA	0.00745	0.00001		0.005
EDTA			0.00007	

*The composition of RM culture medium was obtained from the MV patent. 2000, Nr. a 2000 0154, belonging to Rudic V and Dudnicenco T (10)

CM and BGM had a similar pattern of growth till 10th day of culture. In BGM, maximum cell concentration of 3.053x10^6 cells per ml was reached on 13th day of cultivation whereas in CM maximum growth was limited to 2.599x 10^6cells per ml on 11th day and then decreased slightly. The cell concentration was significantly lower in MBM compared to RM culture medium under the same light intensity.

The maximum cell concentration of 5.77 x 10^6 cells per ml was obtained in BGM on 12th day. MBM recorded a cell growth of 1.827 x10^6 cells per ml on 14th day under the light intensity of 35 µmol photons $m^{-2}s^{-1}$(Yadavalli Fig 1). The cell concentration increased 77.92 times in terms of the initial number in BGM at 35 µmol photons $m^{-2}s^{-1}$. However the maximum cell concentration decreased by only 11% in CM when compared to BGM at 35 µmol photons $m^{-2}s^{-1}$. At 55 µmol photons $m^{-2}s^{-1}$, a significant change was observed in the growth curve after 8th day of culture in all the media (Yadavalli Fig 1). The growth increased by 97.85 times, in terms of initial cell concentration in BGM at 55 µmol photons $m^{-2}s^{-1}$ which is very prominent. Maximum cell concentration of 6.85 x 10^6 cells per ml was observed in BGM on 14th day. The cells in BGM were effective in terms of growth under different light intensities when compared to those grown in other media.



Cell concentrations are expressed as their mean of triplicate samples

Yadavalli Figure 1. Effect of different media on the growth of *C. pyrenoidosa* at different light intensities of 20, 35, 55 µmol photons $m^{-2}s^{-1}$.

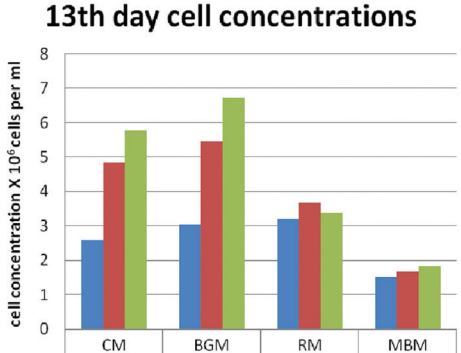
Maximum cell concentrations were observed on 13th day of the culture period in most of the media tested. From the Figure 2 it is observed that the difference in cell concentrations between 20 and 35 µmol photons $m^{-2}s^{-1}$ was higher than the difference between cell concentrations of 35 and 55 µmol photons $m^{-2}s^{-1}$ in CM and BGM. RM and MBM did not have significant change in their cell concentrations at different light intensities. The specific growth rates of *C.pyrenoidosa* in all four media increased with increasing light intensities (Table 2). The doubling time of *C.pyrenoidosa* in all the media was ranging from 50.86-73.72 h. Of the four media, in BGM at 55µmol photons $m^{-2}s^{-1}$ *C.pyrenoidosa* doubled only in 50.86 h. In CM, the doubling time was 53.3 h at the same light intensity, which indicates that the difference in doubling time between CM and BGM at 55µmol photons $m^{-2}s^{-1}$ is insignificant.

From Table 3 it is apparent that as the light intensity increased from 20 to 55µmol photons m^{-2} s^{-1}, the media did not show significant effect on the cell concentration. This can be attributed to lower F_{cal} value than the F_{crit} value at 95% confidence level for all light intensities. The above data also indicate that the effect of media on cell concentration demonstrated an increase with light intensities from 20 to 55 µmol photons $m^{-2}s^{-1}$.

DISCUSSION

The properties of biodiesel from *Chlorella* are comparable to conventional diesel fuel and comply with US standard for biodiesel [14], hence *Chlorella* species have lot of importance in producing biodiesel. One of the most commonly studied species of *Chlorella* is *C.vulgaris* which could produce 10-20 times biomass higher than the maximum production levels reported in the literature when cultured heterotrophically in light and dark conditions [15]. Autotrophic conditions of *Chlorella* species usually provide higher cellular lipid contents and lower biomass when compared with growth in heterotrophic conditions. Yanna Liang et.al demonstrated that *C.vulgaris* provided 38% lipid content in autotrophic conditions and 33% lipid content in heterotrophic conditions but biomass was 6.8 times lesser in autotrophic conditions when compared with heterotrophic conditions [7].

In the present study, comparative studies were carried out by maintaining 16h day and 8h night cycle on different culture media and light intensities to achieve higher cell concentrations of *C. pyrenoidosa* .It was observed that there was an increase in cell concentration with the light intensity up to 55 µmol photons $m^{-2}s^{-1}$ (4000 lux). There is a drastic increase in cell concentration from 20 µmol photons $m^{-2}s^{-1}$ to 35 µmol photons $m^{-2}s^{-1}$ but as light intensity increased from 35 µmol photons $m^{-2}s^{-1}$ to 55 µmol photons $m^{-2}s^{-1}$ the extent of increase was less significant.



	CM	BGM	RM	MBM
20µmol photons m-2s-1	2.586	3.053	3.191	1.521
35µmol photons m-2s-1	4.842	5.455	3.68	1.665
55µmol photons m-2s-1	5.771	6.711	3.362	1.819

Yadavalli Figure 2. 13th day cell concentrations under three different light intensities of *C.pyrenoidosa* grown in different media.

Table 2. Effect of specific growth rates and doubling time with varying light intensities of *C.pyrenoidosa*

S.No	Medium	Light intensity µmol photons m^{-2} s^{-1}	Specific growth rate(µ) d^{-1}	Doubling time(t_d) h
1	CM	20	0.249	67.28
		35	0.303	55.00
		55	0.313	53.30
2	BGM	20	0.261	64.17
		35	0.311	53.72
		55	0.327	50.86
3	RM	20	0.270	62.43
		35	0.279	59.74
		55	0.285	58. 73
4	MBM	20	0.226	73.72
		35	0.232	72.18
		55	0.249	66.63

Martinez et.al had reported that the light saturation constant for *C pyrenoidosa* was 35 µmol photons $m^{-2}s^{-1}$ (2500 lux) in continuous illumination [16]. Our results which were carried out in 16:8 light cycle are not in agreement with the findings of the above study, and the difference could be due to discontinuous illumination or experimental conditions. However, growth is observed even after 35 µmol photons $m^{-2}s^{-1}$ suggesting that significant amount of energy can be saved by conserving 8 h light which can be a cost effective strategy in industrial scale.

As the light intensity increased, biomass productivity increased which is apparent from Figures 1. In BGM and CM, the cell concentration has increased by 2.51 times and 2.46 times respectively by the end of the 14 day cultivation period when the light intensity was increased from 20 to 55µmol photons $m^{-2}s^{-1}$. In RM and MBM the effect of varying light intensities on the cell growth was not significant when compared with CM and BGM.

From the present study it is evident that maximum growth rate of *C.pyrenoidosa* was obtained in BGM followed by CM at 55µmol photons m^{-2} s^{-1}. Change in cell concentration was significant when light intensities were increased from 20 to 35µmol photons m^{-2} s^{-1}. But when light intensities were further increased from 35 to 55µmol photons m^{-2} s^{-1} the increase in cell concentrations was lesser. This study will help for further use of *C.pyrenoidosa* for enhancing lipid productivity.

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Table 3. ANOVA showing the effect of medium composition on cell growth at various light intensities of *C.pyrenoidosa*

S.No	Light Intensity (µmol photons $m^{-2}s^{-1}$)	Variance between media $\frac{\sum_j n_j(\bar{y}_j - \bar{y}_{..})^2}{(k-1)}$	Variance within media $\frac{\sum_{ij}(y_{ij} - \bar{y}_j)^2}{\sum_j (n_j - 1)}$	F_{cal}= Variance between media / within media	$F_{critical}$ (95% confidence level) Where v_1=3,v_2=52
1	20	1.117	0.954	1.18	2.8 - 2.76
2	35	6.186	2.521	2.13	2.8 - 2.76
3	55	8.001	3.269	2.39	2.8 - 2.76

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