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## Lipid Productivity Of *Chlorella pyrenoidosa* In A Customized Lab Scale Photobioreactor Under Stress Conditions

Rajasri Yadavalli\*, Ramgopal Rao S and C. S. Rao

Department of Biotechnology, Sreenidhi Institute of Science and Technology,  
Hyderabad-501301, Andhra Pradesh, India.

\*Corres.author: p.rajasri@gmail.com

**Abstract:** Lab scale studies were conducted in a customized photobioreactor to assess the lipid productivity of *Chlorella pyrenoidosa* under fed-batch mode using 0.025g/l to 0.1 g/l urea and sodium nitrate as nitrogen source in nutrient medium. The light intensities used were 100  $\mu\text{mol m}^{-2}\text{s}^{-1}$  and 135  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . The results indicated maximum lipid productivity of 0.103 g/d.l in BG11 medium with 0.1g/l sodium nitrate in fed batch mode at 135  $\mu\text{mol m}^{-2}\text{s}^{-1}$  whilst 0.1 g/l urea under same conditions has resulted in 0.083g/d.l of lipid productivity. Hence the study demonstrated that sodium nitrate under fed batch mode of cultivation is most suitable for lipid productivity of *C.pyrenoidosa* under high light intensities.

**Keywords:** Light intensity; Nitrate; Urea; Biomass; Photobioreactor.

### Introduction

The ever growing concerns on climate change and global warming necessitates the need for renewable and carbon neutral transport fuels for both ecological and economical sustainability. Microalgae based biodiesel is a potential renewable resource for displacement of liquid transport fuels derived from petroleum<sup>1</sup>. Microalgae are sunlight-driven cellular factories that are easy to culture and require less space for cultivation<sup>2</sup>. They convert carbon dioxide to potential biofuels, valuable bioactive compounds such as carbohydrates, proteins, lipids and pigments<sup>3,4</sup>. Algal lipids offer a great scope as the feedstock of future for sustainable biodiesel production<sup>5,6</sup>.

Even though biodiesel production from algal biomass is pertinent, their relatively high costs are a major obstacle for commercial production. While the

overall lipid productivity determines the costs of the cultivation process, biomass concentration and lipid content affect significantly the downstream processing costs. High lipid cell contents are usually produced by cells under stress, typically nutrient limitation. Eventually, this leads to low biomass and low overall lipid productivity. In this context, a methodology of process optimization that can balance the biomass production and lipid content helps to achieve enhanced lipid productivity.

Previous studies demonstrated that lipid content in some microalgae could be increased by various cultivation conditions such as nitrogen deprivation<sup>5,7</sup>. Nitrogen is known to have a strong influence on the metabolism of lipids and fatty acids in various microalgae. Therefore, nitrogen concentration plays a critical role in enhancing the lipid productivity for bio-fuel production<sup>8</sup>.

It was also observed that higher light intensities influence the biomass productivity<sup>9,10</sup>. A group of scientists claimed that strategies such as intermittent feeding of the nitrogen source and renewal rates used during fed batch cultivation mode improve lipid production. Hence regulation of nutrient feed rates influence the rate of lipid productivity<sup>8</sup>.

This study hypothesizes that these stress conditions might affect the growth rate and lipid productivity of microalgae. However, published data are not available on fed batch strategy for enhancement of lipid production in *Chlorella pyrenoidosa*, a fresh water microalga. Hence this study was undertaken for observing the effect of different nitrogen sources under different light intensities in batch and fed batch modes of cultivation on the biomass and lipid productivities of *C. pyrenoidosa*.

## Materials And Methods

### Algal strain and inoculum preparation

*Chlorella pyrenoidosa* sp. (NCIM NO: 2738) was obtained from National Centre for Industrial Microorganisms (NCIM), Pune, India. Stock culture of *Chlorella pyrenoidosa* was grown photoautotrophically in BG11 media at 28°C under continuous light illumination in four 100 ml borosil flasks. Basal medium was slightly modified for use in this study. Each litre of the BG11 medium

contained NaNO<sub>3</sub>-1.5g, K<sub>2</sub>HPO<sub>4</sub>-0.04g, MgSO<sub>4</sub>•7H<sub>2</sub>O-0.075g, CaCl<sub>2</sub>•2H<sub>2</sub>O-0.036g, Citricacid-0.006g, NaCO<sub>3</sub>-0.02g, H<sub>3</sub>BO<sub>3</sub>-0.00286g, MnCl<sub>2</sub>•4H<sub>2</sub>O-0.00181g, ZnSO<sub>4</sub>•7H<sub>2</sub>O-0.00022g, Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O-0.00039g, CuSO<sub>4</sub>•5H<sub>2</sub>O-0.00008g, Co (NO<sub>3</sub>)<sub>2</sub>•6H<sub>2</sub>O-0.00005g, (NH<sub>4</sub>)<sub>6</sub>Mo7O<sub>24</sub>•4H<sub>2</sub>O-0.003g, Na<sub>2</sub>EDTA-0.00001g. The inoculum was prepared by transferring the cells from stock culture, and incubated aseptically in a 1000 ml flask containing 700 ml of fresh BG11 media under continuous illumination of 34 μmol m<sup>-2</sup>s<sup>-1</sup> at 28°C for four days on an orbital shaker set at 120 rpm. Light intensity was measured with a LUX meter(LM-52-780) and illumination was provided by standard cool white fluorescent lamps. A 4 day old culture was used as inoculum at 10% volume for the preparation of stock cultures.

### Stock culture

Stock culture was prepared in 1000 ml flasks by inoculating 39 ml of the 4 day old seed culture into 8 flasks, each containing 700 ml of sterilized, fresh BG11 media of varying concentrations of urea and sodium nitrate, respectively. Both low and high concentrations of urea and sodium nitrate were tested and denoted by LU, HU, LN, and HN (Table 1). The flasks were incubated for 4 days at 28°C in a continuous light illumination of 110 μmol m<sup>-2</sup>s<sup>-1</sup>, on an orbital shaker set at 120 rpm. These cultures were used for inoculation into the photo-bioreactors.

**Table 1:** Concentration of Urea and Nitrate as Nitrogen sources used in the present study

S. No	Nitrogen Source Concentration (g/l)	Medium Label
1	0.025	LU-1
		LN-1
2	0.05	LU-2
		LN-2
3	0.1	LU-3
		LN-3
4	0.15	LU-4
		LN-4
5	0.5	HU-5
		LN-5
6	1.0	HU-6
		LN-6
7	1.5	HU-7
		LN-7
8	2.0	HU-8
		LN-8

LU represents Low Urea concentration, LN represents Low Sodium nitrate concentration, HN represents High Sodium nitrate concentration and HU represents High Urea concentration.

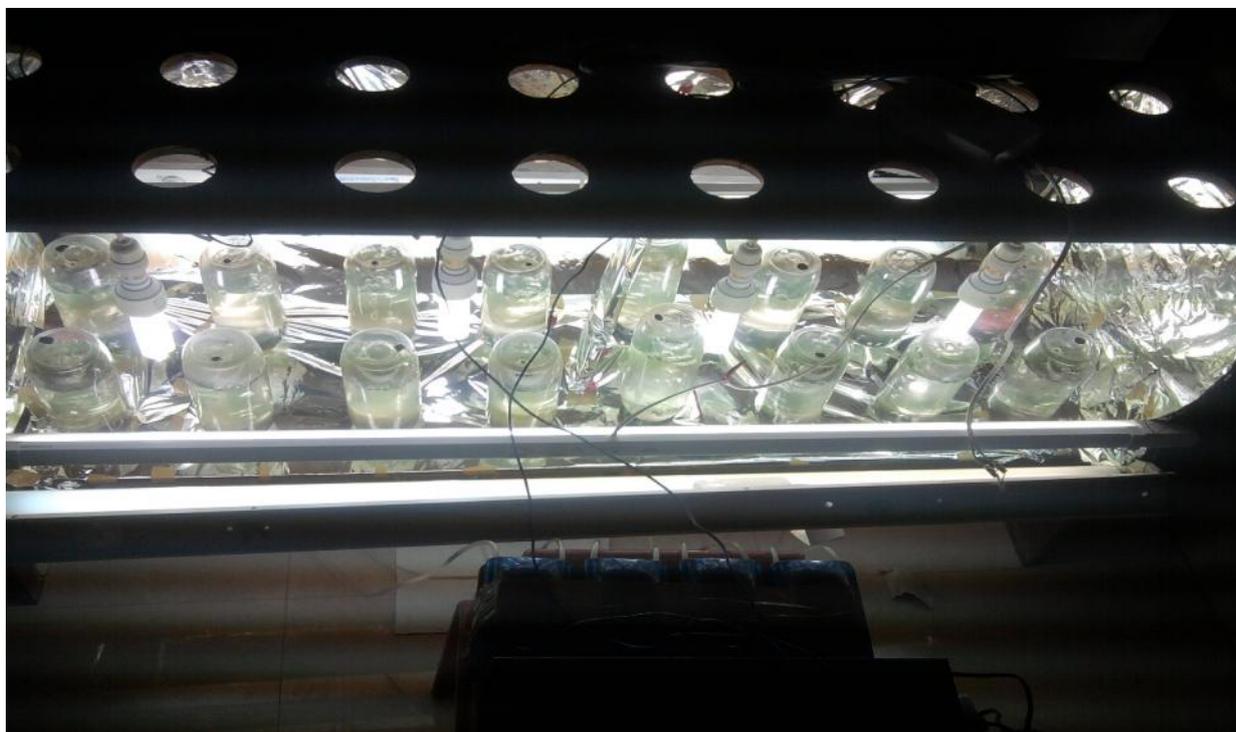
### ***Photobioreactor assembly and cultivation conditions***

Figure 1 illustrates the lab scale customized photo-bioreactor set up used in the present study. *C. pyrenoidosa* was cultured in commercially available polycarbonate refrigerator bottles with 700 ml of working volume placed at  $26 \pm 1$  ° C under continuous, cool white, fluorescent lights. Two rows with adequate provision for lighting 8 bottles on each side were fabricated with wood. All the 16 bottles were arranged in an inverted position on this supporting platform. The platform was provided support for illumination purposes which was fulfilled by high intensity tube lights and suspended CFL lamps delivering an overall light intensity of  $110 \mu\text{mol m}^{-2}\text{s}^{-1}$  and  $135 \mu\text{mol m}^{-2}\text{s}^{-1}$  in two different setups. This experimental setup is a power-saving method and useful in plant tissue and algal culture laboratories. Silver-tinted polyester film was fixed on all sides of culture racks to reflect the light. This method is simple, inexpensive and saves 50% electric energy by reducing the number of lights or thin wattage, thus contributing to energy conservation<sup>11</sup>.

A test run was performed using 4 inverted bottles pumped with air and certain difficulties like back flow, intermixing, wall growth and settling of cells were encountered. These problems were overcome by using separate air pumps for each pair of bottles and plain, transparent, wide mouthed refrigerator bottles were used instead of soda bottles. By eliminating all the erroneous factors, a sustained and orderly growth conditions were achieved. Continuous aeration was provided with intermittent CO<sub>2</sub> supply to the reactors at regular intervals of 3 h and at a rate of 1.0 l/min. Air was humidified before it is sent into reactors for reducing evaporation losses. Sampling was done daily and the optical density of each sample was recorded for every 24 hours. Sampling was done on daily basis and the optical density of the samples was recorded.

### ***Experimental design of Fed batch mode cultivation***

The effects of urea and sodium nitrate feed concentration on microalgal growth and lipid production was investigated in fed-batch cultivation. The initial biomass concentration at the inoculation time in all the runs was 0.1 g/l, and cultures were incubated for 8 days. The urea and sodium nitrate were added on fourth day of the culture period.



**Figure 1: Customized Laboratory scale Photo bioreactor set up**

### **Biomass Dry Cell Weight (DCW) measurement**

Biomass content was determined by measuring the optical density of samples at 600 nm (OD<sub>600</sub>). The conversion factor was established by plotting OD<sub>600</sub> versus DCW of a series of samples of different biomass concentrations. Samples were diluted by appropriate ratios to ensure that the measured OD<sub>600</sub> values were in the range of 0.2–0.9. DCW of a sample was determined gravimetrically after drying and the algal cells were collected from samples with centrifugation (3,000×g, 10 min) and washed with water. Linear regression equation obtained was

$$y = 1.038557658 \cdot 10^{-1} x - 7.295013686 \cdot 10^{-4} \text{ and } r = 9.839458706 \cdot 10^{-1}$$

where y is DCW of algal cells and x is optical density at 600nm.

### **Extraction and estimation of lipid productivity**

Extraction of lipid was done by a rapid method of total lipid extraction and purification<sup>12</sup>. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were washed once with distilled water and centrifuged again. The pellet was then subjected to wet weight estimation and dried in oven for 2 h at 80°C. For 1 g of algal biomass, 2 ml of methanol and 1 ml of chloroform was added and kept for 18 hours at 25°C. The mixture was agitated in vortex for 2 min. 1 ml of chloroform was again added and the mixture was shaken vigorously for 1 min. 1ml of distilled water was added and the mixture was mixed in a vortex again for 2 min. The layers were separated by centrifugation for 10 min at 2000 rpm. The lower layer was separated and the procedure was again repeated with the pellet. The two supernatants collected were allowed to stand for 2 h. Lower organic layer with the lipids was transferred to a clean pre-weighed vial (W1). Evaporation was carried out in hot air oven at 80°C for 50 min. The weight of the vial was again recorded (W2). Lipid yield was calculated by subtracting W1 from W2. The lipid productivity was calculated by the following equation:

$$P_{\text{lipid}} = \frac{C_{\text{lipid}} \times \text{DCW}}{\text{Time}}$$

Where P<sub>lipid</sub> is lipid productivity in g l<sup>-1</sup> day<sup>-1</sup>, C<sub>lipid</sub> is lipid content of cells or lipid yield of the cells in g/g, DCW is dry cell weight g/l, and Time is the cultivation period in days.

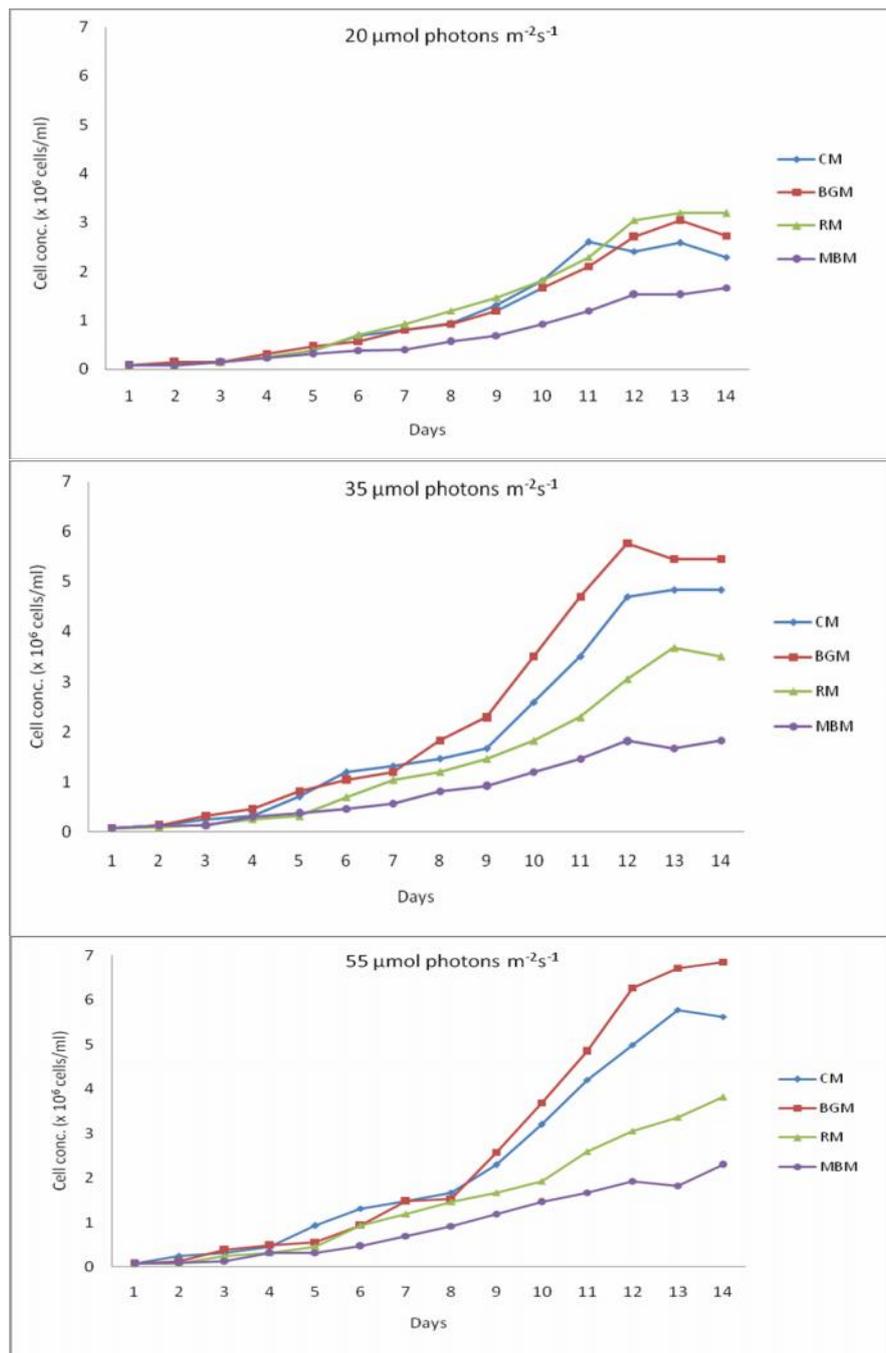
## **Results And Discussion**

### **Growth Characteristics of *C.pyrenoidosa***

Our previous studies have shown that *C.pyrenoidosa* has shown that as light intensity is increasing the cell growth was increasing. Four media (Mod. Basal Medium, Rudic's Medium, Chlorella Medium, BG11 Medium) each with 20, 35 and 55 μmol photons m<sup>-2</sup>s<sup>-1</sup> light intensities resulting in twelve different combinations were studied (Fig 2). As light intensity increased from 20 μmol photons m<sup>-2</sup>s<sup>-1</sup> to 55 μmol photons m<sup>-2</sup>s<sup>-1</sup>, 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<sup>6</sup> cells per ml was obtained in BG11 medium at 55 μmol photons m<sup>-2</sup>s<sup>-1</sup> corresponding to a specific growth rate (μ) of 0.327d<sup>-1</sup> with a least doubling time (t<sub>d</sub>) of 50.86h.

### **Fed batch mode**

Literature suggests that a 22 % drop in lipid yield was observed in *Nannochloris sp* when nitrate concentration increased from 0.9 mM/l to 9.9 mM/l (Takagi M et al.2000). Our study demonstrated that lipid yield decreased with an increase in nitrogen concentration which is in correlation with the above findings. Maximum DCW obtained by *C.pyrenoidosa* in 8 days in fed batch mode was 2.870 g/l in HU-8 medium and 3.061 g/l in HN-8 medium at 135 μmol m<sup>-2</sup>s<sup>-1</sup>, respectively (Table 2). At the end of 8<sup>th</sup> day the DCW showed an increase of 378% in HU-8 medium at 135 μmol m<sup>-2</sup>s<sup>-1</sup> and by 329% at 110 μmol m<sup>-2</sup>s<sup>-1</sup> respectively (Table 2) which is supported by our earlier studies where we observed that light intensity is directly proportional to the growth of *C.pyrenoidosa*.<sup>10</sup>.



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

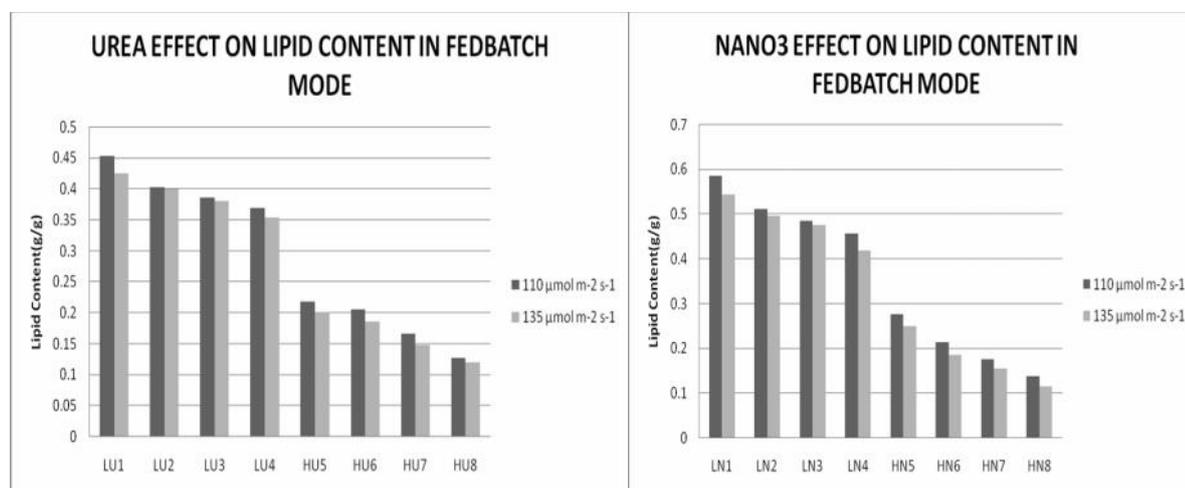
**Table 2: Effect of Urea and Sodium nitrate on Biomass DCW and LY in Fed-batch mode after 8 days of cultivation**

S. No	Nitrogen Conc (g/l)	Biomass Dry Cell Weight (g/l) with Urea		Lipid Yield (g/g) with Urea		Biomass Dry Cell Weight (g/l) with Sodium nitrate		Lipid Yield (g/g) with Sodium nitrate	
		at 110 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 135 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 110 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 135 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 110 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 135 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 110 $\mu\text{mol m}^{-2}\text{s}^{-1}$	at 135 $\mu\text{mol m}^{-2}\text{s}^{-1}$
1	L-1	0.854±0.003	1.200±0.030	0.453±0.010	0.425±0.005	1.003±0.148	1.295±0.025	0.585±0.007	0.544±0.014
2	L-2	1.301±0.021	1.530±0.010	0.403±0.005	0.399±0.007	1.390±0.100	1.554±0.026	0.511±0.012	0.496±0.015
3	L-3	1.405±0.022	1.725±0.035	0.386±0.005	0.380±0.009	1.520±0.110	1.733±0.013	0.484±0.014	0.475±0.008
4	L-4	1.399±0.034	1.820±0.030	0.369±0.011	0.354±0.012	1.570±0.150	1.865±0.025	0.456±0.011	0.418±0.01
5	H-5	2.169±0.055	2.605±0.015	0.218±0.007	0.201±0.010	2.339±0.119	2.649±0.039	0.277±0.008	0.249±0.008
6	H-6	2.404±0.020	2.670±0.020	0.206±0.005	0.186±0.007	2.493±0.077	2.770±0.080	0.214±0.007	0.185±0.015
7	H-7	2.480±0.040	2.750±0.030	0.166±0.006	0.148±0.009	2.540±0.051	2.855±0.065	0.177±0.011	0.156±0.009
8	H-8	2.576±0.006	2.870±0.010	0.127±0.003	0.120±0.004	2.715±0.135	3.061±0.151	0.139±0.006	0.116±0.014

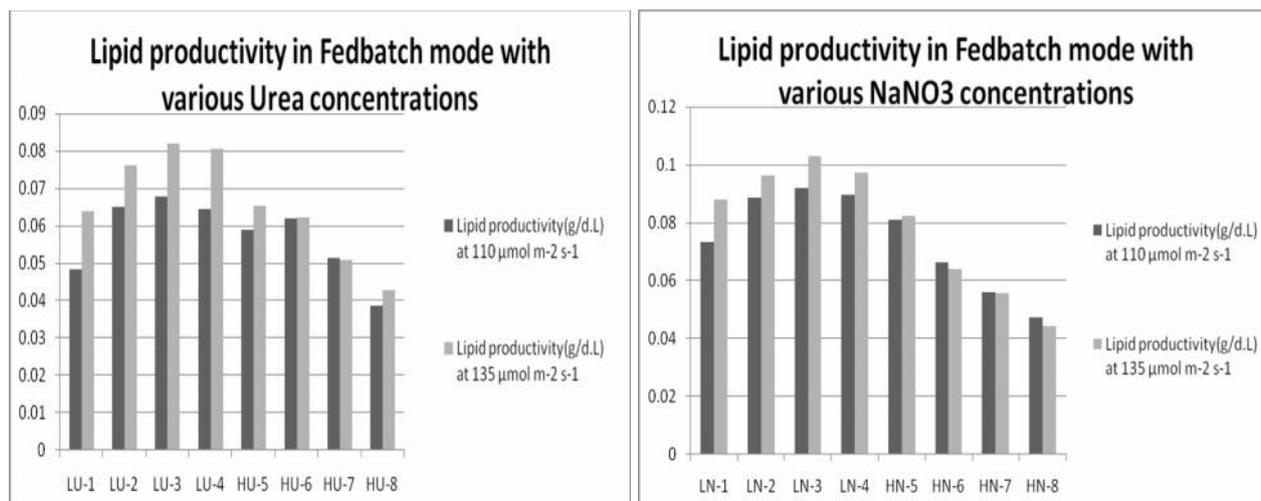
The values in the above table are taken with triplicate samples and are represented as Mean  $\pm$  S.E. L-1 to H-8 in the second column represents various Urea and Sodium nitrate concentrations from lower to higher concentrations.

In HN-8 medium a 410% increase in DCW was observed at 135  $\mu\text{mol m}^{-2}\text{s}^{-1}$  from initial to final day and it increased by 352% in 110  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . However, there was a slight increase of 6% in DCW in HN-8 medium when compared to HU-8 medium at 135  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Our results are in accordance with few studies which indicated that there is a loss of biomass when *Botryococcus sp.* and *Scenedesmus obliquus* were exposed to nitrogen deficient conditions<sup>13,14</sup>. In fed batch mode, sodium nitrate was best source of nitrogen when compared with urea for *C. pyrenoidosa* biomass production.

As shown in Fig. 3A and 2B, lipid yield increased with reducing nitrogen concentrations in 3th medium. Literature suggests that nitrogen is the most common nutritional-limiting factor triggering lipid accumulation in microalgal cells<sup>15,16,17</sup>. In nitrogen limiting media, the lipid content usually increases in algae due to less susceptibility of lipid-synthesizing enzymes for disorganization than carbohydrate synthesizing enzymes due to nitrogen deprivation. Higher lipid yield of algae reduces lipid production costs and is an important parameter that determines the economy of algae biodiesel production.



**Figure 3A and 3B: The effect of Urea and Sodium nitrate on lipid yield in fed batch modes at 110 and 135  $\mu\text{mol m}^{-2}\text{s}^{-1}$**



**Figure 4A and 4B: The effect of Urea and Sodium nitrate on lipid productivity in fed batch mode at 110 and 135  $\mu\text{mol m}^{-2} \text{s}^{-1}$**

In lowest urea concentration a maximum lipid yield of 0.453 g/g was obtained at 110  $\mu\text{mol m}^{-2} \text{s}^{-1}$  while at 135  $\mu\text{mol m}^{-2} \text{s}^{-1}$  it reduced to 0.425 g/g (Fig. 3A). Maximum lipid productivity of 0.082 g/d.l and 0.081 g/d.l was obtained at 135  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in LU-3 and LU-4 media respectively (Fig. 4A and 4B).

In the present study, the increased lipid cell content in lower sodium nitrate concentration could be due to the low initial nitrogen concentrations in the medium will exhaust at low cell density since light can penetrate enough, resulting in enhanced metabolic flux from photosynthesis which might be channelled to lipid accumulation on a unit biomass basis. This observation suggests that cells accumulate large quantities of chlorophyll molecules when nitrogen source was abundantly available. Upon exhaustion of external nitrogen sources, the cells start to utilize chlorophyll as an intracellular nitrogen source.

At 135  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , lipid productivity was maximum viz, 0.103 g/d.l, 0.097 g/d.l, 0.0963 g/d.l in LN-3, LN-4, and LN-2 media, respectively (Fig. 4A and 4B). In LN-3 medium, maximum lipid productivity of 0.092 g/d.l was observed at 110  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Our results are in agreement with other such studies which employed intermittent addition of nitrate for fed-batch cultivation of green algae and cyanobacteria to enhance the lipid yield<sup>18,19</sup>.

## Conclusion

*Chlorella pyrenoidosa* growth was directly proportional to the concentration of nitrate in the medium. As nitrate source is increased in the medium enhancement in biomass concentration was observed. It was also observed that lipid yield increased in nitrogen limiting conditions. In the present study it was observed that lipid productivity increased with an increase in light intensities and this could be due to the increase in biomass concentration. Maximum lipid productivity (0.103 g/d.l) was obtained at 135  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in LN-3 medium in fed batch mode of cultivation. *C. pyrenoidosa* prefers sodium nitrate as nitrogen source than urea in photoautotrophic conditions where it exhibited lipid enhancement of *C. pyrenoidosa*. We conclude that fed batch mode effectively enhanced the lipid productivity of *C. pyrenoidosa* when sodium nitrate was selected as nitrate source making it an important option in the cultivation of microalgae.

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