Rajasri Yadavalli, Ramgopal Rao S, C. S. Rao / International Journal of Engineering Research and Applications (IJERA) ISSN: 2248-9622 www.ijera.com
Vol. 2, Issue 3, May-Jun 2012, pp.2446-2453

Lipid Accumulation Studies In *Chlorella Pyrenoidosa* Using Customized Photobioreactor- Effect of Nitrogen Source, Light Intensity and Mode of Operation

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ABSTRACT

Lipid productivity of *Chlorella pyrenoidosa* was studied in a customized laboratory scale photobioreactor. Lipid yield increased when *C. pyrenoidosa* was subjected to stress conditions like different nitrogen sources, light intensities and modes of cultivation. It was observed that the growth rate of *C. pyrenoidosa* was directly proportional to light intensity and nitrogen concentrations. Of the two nitrogen sources tested, sodium nitrate proved better than urea in terms of lipid yield. The study also demonstrated that at lower nitrogen concentrations fed batch mode of cultivation resulted in maximum lipid productivity of 0.103 g/d.l at 135 µmol $m^{-2}s^{-1}$ when compared to batch mode.

Keywords: Modes of cultivation, Light intensities, Biomass, Lipid productivities, *C. pyrenoidosa* Photobioreactor

1. INTRODUCTION

Rapid depletion of fossil fuels poses a major challenge to mankind. 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. Alternate energy resources like biodiesel are commercially produced currently from plant and animal oils, but not from microalgae which are photosynthetic and ever-present in any aquatic ecosystem. Microalgae are sunlight-driven cellular factories that are easy to culture and require less space for cultivation [1]. They convert carbon dioxide to potential biofuels, valuable bioactive compounds such as carbohydrates, proteins, lipids and pigments [2, 3]. Research indicates that much higher growth rates and photosynthetic efficiencies can be achieved with microalgae than conventional terrestrial plants. Algal lipids offer a great scope as the feedstock of future for sustainable biodiesel production [1, 4, 5, 6]. Microalgae based biodiesel is a potential renewable resource for displacement of liquid transport fuels derived from petroleum [7].

Even though biodiesel production from algal biomass is pertinent, their relatively high costs are a major obstacle for commercial production. Biomass concentration, increasing lipid content, and overall lipid productivity hold the key for economic feasibility of algal oil for biodiesel production. While the overall lipid productivity determines the costs of the cultivation process, biomass concentration and lipid content affect significantly the downstream processing costs. In this context, process optimization that can maneuver the algal biochemical production, fast growth and good environmental adaptability helps to achieve environmental and economic sustainability. An ideal process should produce the highest productivity of algae with enhanced cellular lipid content. Unfortunately, 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. Recently it was reported that a maximum lipid yield of 0.40 g/l for autotrophic *Scenedesmus dimorphus* from the 1.8 g/l urea medium, and 5.89 g/l for heterotrophic *Chlorella protothecoides* from the 2.4 g/l nitrate medium were achieved [8]. Previous studies have demonstrated that lipid content in some microalgae could be increased by various cultivation conditions such as nitrogen deprivation [4, 9, 10], high light intensity [11].

Many microalgae respond to stress conditions by significantly increasing the lipid content capacities, by 30% - 60% of the dry cell weight. Amongst these factors, nitrogen is known to have a strong influence on the metabolism of lipids and fatty acids in various microalgae. In addition, nitrogen is easy to manipulate and is cost effective compared to other factors. Therefore, nitrogen concentration plays a critical role in enhancing the lipid productivity for bio-fuel production [10]. A few microalgal species, including some *Chlorella* species [5, 9, 12, 13], *Nannochloris* sp. [10], *Neochloris oleoabundans* [14], and *Botryococcus braunii* [15, 16], have been reported to accumulate large quantities of lipids in cells under stress conditions.

Regulation of nutrient feed rates to increase productivity can be performed by fed-batch cultivation. Researchers reported that maximum lipid productivity was obtained in a semi-continuous culture when compared with those in the batch and fed-batch cultivations of *Chlorella* sp., the marine micro algae used with urea as a nitrogen limitation [17]. However, published data are not available on fed batch strategy for enhancement of lipid production in *Chlorella pyrenoidosa*, a fresh water microalga. Few reports have demonstrated that strategies such as intermittent feeding

of the nitrogen source and renewal rates used during fed batch cultivation mode improve lipid production [10].

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*. This study hypothesizes that these stress conditions might affect the growth rate and biochemical composition of microalgae.

2. MATERIALS AND METHODS

2.1 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^{o} C under continuous light illumination in four 100 ml borosil flasks. Basal medium [18] was slightly modified for use in this study. Each litre of the BG11 medium contained $NaNO_3$-1.5g, K_2HPO_4-0.04g,$MgSO_4$•$7H_2O$-0.075g,$CaCl_2$•$2H_2O$-0.036g, Citricacid-0.006g,$NaCO_3$-0.02g,H_3BO_3-0.00286g, $MnCl_2$•$4H_2O$-0.00181g,$ZnSO_4$•$7H_2O$-0.00022g, Na_2MoO_4•$2H_2O$-0.00039g, $CuSO_4$•$5H_2O$-0.00008g, $Co(NO_3)_2$•$6H_2O$-0.00005g,$(NH_4)_6Mo7O_{24}.4H_2O$-0.003g, Na_2EDTA-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 $m^{-2}s^{-1}$ at 28^{o}C for four days on an orbital shaker set at 120 rpm. A 4 day old culture was used as inoculum at 10% volume for the preparation of stock cultures.

2.2 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^{o}C in a continuous light illumination of 110 µmol $m^{-2}s^{-1}$, on an orbital shaker set at 120 rpm. These cultures were used for inoculation into the photo-bioreactors.

2.3 Photobioreactor Assembly and Cultivation Conditions

Fig 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 ±1 ^{o}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 µmol $m^{-2}s^{-1}$ and 135 µmol $m^{-2}s^{-1}$ 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 [19]. 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_2 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 fed batch mode of operation nitrogen source was added on fourth day to the first row bottles in the same initial concentrations. Sampling was done on daily basis and the optical density of the samples was recorded for three more days.

2.4 Experimental Design of Batch Mode Cultivation

The effects of urea and sodium nitrate concentration on microalgal growth and lipid production were investigated, and cultivations were carried out in reactors containing BG11 medium with above mentioned nitrogen concentrations (Table 1).The initial biomass concentration at the inoculation time in all the runs was 0.1 g/l, and cultures were incubated for 8 days.

2.5 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.

2.6 Biomass Dry Cell Weight (DCW) Measurement

Biomass content was determined by measuring the optical density of samples at 600 nm (OD_{600}). The conversion factor was established by plotting OD600 versus DCW of a series of samples of different biomass concentrations. Samples were diluted by appropriate ratios to ensure that the measured OD_{600} values were in the range of 0.2–0.9. DCW of a sample was determined in 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 $y = 1.038557658 \cdot 10^{-1} x - 7.295013686 \cdot 10^{-4}$ and $r = 9.839458706 \cdot 10^{-1}$ where y is DCW of algal cells and x is optical density at 600nm.

2.7 Extraction and Estimation of Lipid Productivity

Extraction of lipid was done by a rapid method of total lipid extraction and purification [20]. 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:

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

Where P_{lipid} is lipid productivity in g l^{-1} day^{-1}, C_{lipid} 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.

3. RESULTS AND DISCUSSION

3.1 Batch mode

Biomass concentration increased with an increase in nitrogen concentration in the medium [21, 22]. The DCW of *C. pyrenoidosa* was found to be more when sodium nitrate was used as nitrogen source compared to urea indicating that sodium nitrate is the most preferred nitrogen source in batch mode. In batch mode, maximum dry cell weight (DCW) of 2.805 g/l was obtained in 8 days culture in HU-8 medium at 135 µmol $m^{-2}s^{-1}$ and 2.846 g/l in HN-8 medium at 135 µmol $m^{-2}s^{-1}$ (Table 2). The DCW demonstrated a linear and positive correlation with increased light intensity.

The DCW increased by about 332% at 110 µmol $m^{-2}s^{-1}$ in HU-8 medium, and by 200% in LU-1 medium, compared to the initial day DCW. Similar trends were observed in DCW even at 135 µmol $m^{-2}s^{-1}$ for HU-8 medium and the DCW increased by 44% at 135 µmol $m^{-2}s^{-1}$ when urea concentration was increased. In HN-8 medium the DCW increased by 370% at 135 µmol $m^{-2}s^{-1}$ and by 330% at 110 µmol $m^{-2}s^{-1}$ when compared to initial biomass. Our results are in accordance with few studies which indicated that there is a loss of biomass when *Botrycococcus sp. and Scenedesmus obliquus were* exposed to nitrogen deficient conditions [23, 24].

As shown in Fig 2A and 2B, lipid yield increased with reducing nitrogen concentrations in the medium. Literature suggests that nitrogen is the most common nutritional-limiting factor triggering lipid accumulation in microalgal cells [14, 25, 26, 27]. 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**.**

Maximum lipid yield of 0.534 g/l was reported in LN-1 medium at 110 µmol $m^{-2}s^{-1}$. Further, it was also observed that as light intensity increased the lipid yield exhibited a slight reduction. In LU-1 medium, maximum lipid yield of 0.4 g/l was reported at 110 µmol $m^{-2}s^{-1}$. The lipid yield decreased by 240% as urea concentration increased in the medium from 0.025% to 2% at 110 µmol $m^{-2}s^{-1}$ (Table 2). Similar trend was observed in the lipid yield even at 135 µmol $m^{-2}s^{-1}$. The results suggest that lipid yield of *C. pyrenoidosa* decreases in the presence of urea even with increased light intensity.

At 110 µmol $m^{-2}s^{-1}$, lipid productivity was 0.0808 g/d.l and 0.0806 g/d.l in LN-3 and LN-2 media, respectively. A maximum lipid productivity of 0.093g/d.l, 0.091g/d.l and 0.0908g/d.l was obtained in LN-3, LN-4 and LN-2 media at 135 µmol $m^{-2}s^{-1}$ respectively. In batch mode, the maximum lipid productivity obtained in LU-2 and LU-3 media 135 µmol $m^{-2}s^{-1}$ was 0.0677 g/d.l and 0.068 g/d.l respectively. At 110 µmol $m^{-2}s^{-1}$, maximum lipid productivity of 0.0635 g/d.l and 0.0626 g/d.l was obtained in LU-2 and LU-3 media respectively (Fig.3A). Figure 3b showed that sodium nitrate was proved as better nitrogen source than urea in achieving more lipid yield .The lipid yield decreased by 318% at 110 µmol $m^{-2}s^{-1}$ and by 440% at 135 µmol $m^{-2}s^{-1}$ from lowest to highest nitrogen concentrations in the medium (0.025% to 2%). There was a sudden decrease of 85% in lipid yield at 110 µmol $m^{-2}s^{-1}$ when sodium nitrate concentration in the medium was increased from 0.15% to 0.5% and that of 88% decrease in lipid yield at 135 µmol $m^{-2}s^{-1}$.

In the present study, the increased lipid cell content in lower sodium nitrate concentration could be due to the following reasons. 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 channeled 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.

3.2 Fed Batch Mode

Literature suggests that limited feeding of potassium nitrate for fed-batch cultivation of *Nannochloris* sp successfully increased the intracellular lipid yield by 30% over that of batch culture [10]. Our study demonstrated that lipid yield decreased with an increase in nitrogen concentration which is in correlation with the above findings. These observations are also in accordance with another report in which 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 [28]. 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 $m^{-2}s^{-1}$, respectively (Table 3). As in the case of batch mode, the biomass concentration increased with an increase nitrogen concentration and the DCW increased with increased light intensities. The DCW increased by 12% in fed batch compared to batch mode in 2% HU-8 medium when the light intensity increased from 110 to 135 µmol $m^{-2}s^{-1}$. At the end of 8^{th} day the DCW showed an increase of 378% in HU-8 medium at 135 µmol $m^{-2}s^{-1}$ and by 329% at 110 µmol $m^{-2}s^{-1}$ respectively (Table 3) which is supported by our earlier studies where we observed that light intensity is directly proportional to the growth of *C. pyrenoidosa*[29].

In HN-8 medium a 410% increase in DCW was observed at 135 µmol $m^{-2}s^{-1}$ from initial to final day and it increased by 352% in 110 µmol $m^{-2}s^{-1}$. However, there was a slight increase of 6% in DCW in HN-8 medium when compared to HU-8medium at 135 µmol $m^{-2}s^{-1}$. Like in batch mode sodium nitrate was best source of nitrogen when compared with urea for *C. pyrenoidosa* biomass production.

In lowest urea concentration a maximum lipid yield of 0.453 g/g was obtained at 110 µmol $m^{-2}s^{-1}$ while at 135 µmol $m^{-2}s^{-1}$ it reduced to 0.425 g/g. As in batch mode as urea concentration increased lipid yield decreased at both the light intensities. As indicated in figure 3a lipid yield decreased by 7% in lowest urea concentration as light intensity increased from 110 to 135 µmol $m^{-2}s^{-1}$. Maximum lipid productivity of 0.082 g/d.l and 0.081 g/d.l were obtained at 135 µmol $m^{-2}s^{-1}$ in LU-3 and LU-4 media respectively. A 20% increase in lipid productivity was observed when urea was used as nitrogen source in the medium at 135 µmol $m^{-2}s^{-1}$ in fed batch compared to batch mode (Fig. 3A and 3B).

Interestingly in both modes of cultivation (batch and fed batch) maximum lipid productivity was obtained in LU-3 medium. While maximum lipid yield of 0.585 g/g was observed in LN-1 medium at 110 µmol $m^{-2}s^{-1}$, it decreased by 7% from 110 to 135 µmol $m^{-2}s^{-1}$(Table 3). Similar to batch mode, the lipid yield decreased as nitrate concentration is increased when sodium nitrate is used as nitrogen source (Fig.2B). In lowest nitrogen source, a 10 % increase in lipid yield was observed at 110 µmol $m^{-2}s^{-1}$ from batch to fed batch mode of operation.

At 135 µmol $m^{-2}s^{-1}$, 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. In LN-3 medium, maximum lipid productivity of 0.092 g/d.l was observed at 110 µmol $m^{-2}s^{-1}$. Interestingly here too LN-3 resulted in highest lipid productivity as in batch mode. Also it is apparent from figures 3A and 3B that lipid productivity increased from batch to fed batch at both light intensities. 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 [30].

4. 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 µmol $m^{-2}s^{-1}$ 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.

ACKNOWLEDGEMENTS

We thank the Management of Sreenidhi Institute of Science and Technology (SNIST) for their financial support in carrying out this in-house project.

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Figure 1 : Customized laboratory scale photo bioreactor set up

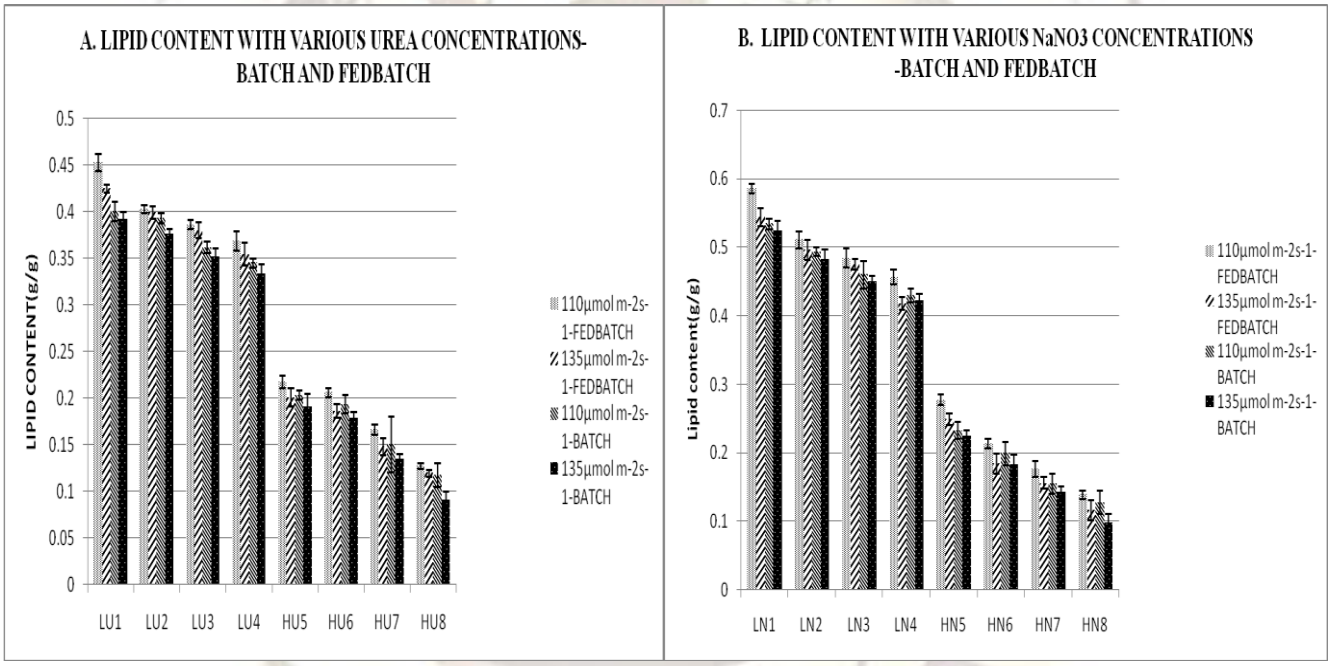

FIG. 2A and 2B.

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

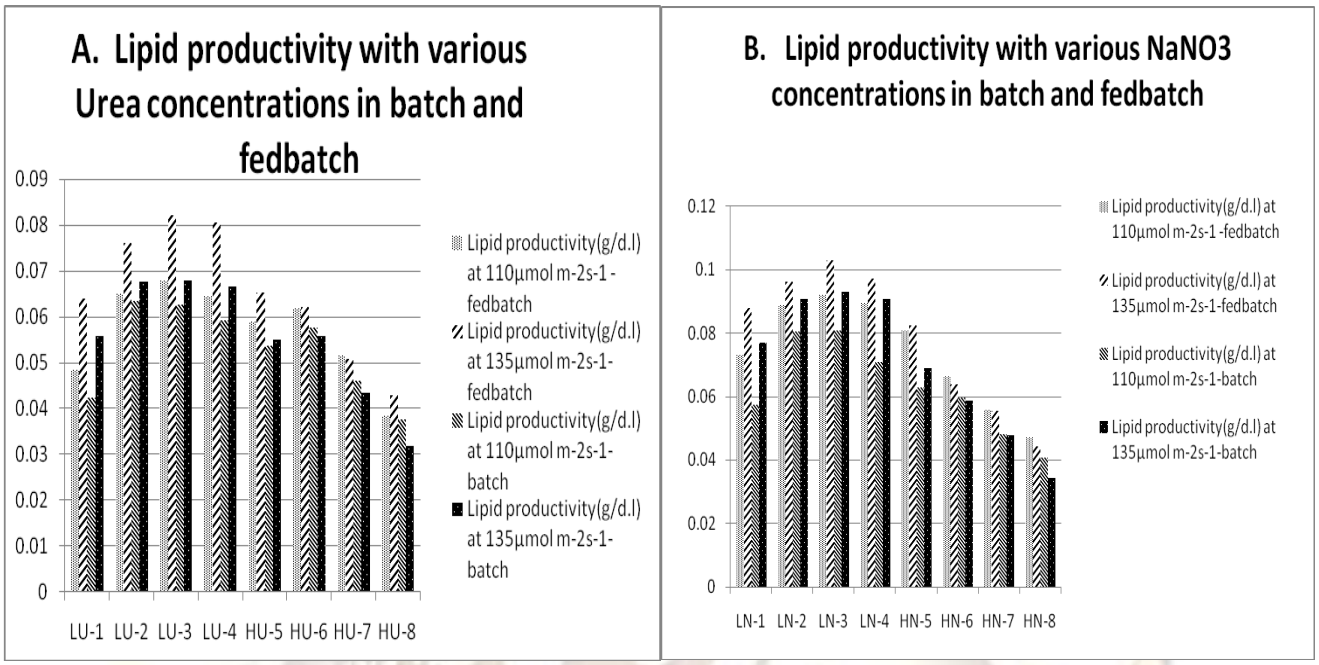

Figure 3A and 3B: The effect of Urea and Sodium nitrate on lipid productivity in batch and fed batch modes at 110 and 135 µmol $m^{-2}s^{-1}$

TABLE 1: Concentration of Urea and Nitrate as nitrogen source 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.

TABLE 2: Effect of Urea and Sodium nitrate on biomass DCW and LY in 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 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$	at 110 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$	at 110 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$	at 110 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$
1	L-1	0.845±0.011	1.140±0.020	0.400±0.010	0.3915±0.008	0.860±0.002	1.175±0.035	0.534±0.008	0.524±0.011
2	L-2	1.295±.045	1.440±0.040	0.393±0.006	0.376±0.005	1.310±0.006	1.505±0.055	0.494±0.007	0.483±0.015
3	L-3	1.385±0.075	1.550±0.010	0.362±0.007	0.351±0.009	1.405±0.001	1.647±0.090	0.460±0.020	0.450±0.02
4	L-4	1.370±0.090	1.595±0.025	0.345±0.005	0.3335±0.010	1.394±0.001	1.721±0.122	0.430±0.010	0.423±0.010
5	H-5	2.115±0.035	2.300±0.200	0.203±0.005	0.191±0.013	2.170±0.0003	2.465±0.145	0.233±0.013	0.224±0.011
6	H-6	2.385±0.065	2.495±0.015	0.194±0.010	0.179±0.006	2.404±0.0003	2.583±0.092	0.199±0.017	0.183±0.007
7	H-7	2.450±0.040	2.580±0.010	0.150±0.030	0.1345±0.006	2.484±0.0025	2.673±0.099	0.155±0.015	0.143±0.011
8	H-8	2.560±0.020	2.805±0.015	0.118±0.013	0.091±0.009	2.576±0.0004	2.846±0.036	0.128±0.018	0.097±0.027

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

TABLE 3: 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 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$	at 110 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$	at 110 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$	at 110 µmol $m^{-2}s^{-1}$	at 135 µmol $m^{-2}s^{-1}$
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 ± S.E. L-1 to H-8 in the second column represents various Urea and Sodium nitrate concentrations from lower to higher concentrations