Journal of Algal Biomass Utilization
J. Algal Biomass Utln. - Copyright © PHYCOSPECTRUM
ISSN: 2229 – 6905

Scenedesmus dimorphus and *Scenedesmus quadricauda* : two potent indigenous microalgae strains for biomass production and CO_2 mitigation - A study on their growth behavior and lipid productivity under different concentration of urea as nitrogen source.

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Abstract

Microalgae are fast growing biological systems which are capable of fixing high amount of carbon dioxide through photosynthesis to produce biomass**.** The two fresh water microalgae strains, *Scenedesmus dimorphus* and *Scenedesmus quadricauda* were selected for this study. Both the algal strain were cultivated in different concentrations of urea as nitrogen source in the growth medium. The concentration of urea at which maximum growth rate in terms of biomass and lipid productivity obtained was at 0.1g/L urea in case of both the strains. The maximum increase in biomass per day and lipid content for *Scenedesmus dimorphus* was found to be 1.523 mg/L/day and 34% in terms of dry cell weight with a specific growth rate of 0.54 /day of and for *Scenedesmus quadricauda* it was 1.266 mg/L/day and 31% in terms of dry cell weight with a specific growth rate 0.392 /day. It was observed that when urea concentration was increased beyond 0.1g/L, the biomass and cell number decreased gradually and both strains showed a lower lipid productivity.

Keywords – lipid, urea, biomass, biofuel

Introduction

Microalgae are a group of fast growing unicellular or simple multicellular micro organism which have the ability to fix CO_2 while capturing solar energy with an efficiency 10 to 50 times greater than that of terrestrial plants and higher biomass production compared to energy crops (Wang *et al.*,2008). The main environmental factors influencing micro algal growth and chemical composition are light, nutrients, temperature and pH (Rousch *et al.*, 2003). In recent years microalgae cultivation has received much attention on account of their utility as a feasible CO_2 sequestration technology (Ono and Cuello,2006). Micro algae have several advantages, including higher photosynthetic efficiency as well as higher growth rates and higher biomass production compared to other energy crops. Several micro algae strains have been reported to have the ability to accumulate large quantities of lipids. It is also observed that nitrogen limitation leads to an increase of lipid content in some chlorella strains (Reiten *et al.*, 1994).Previous studies have confirmed that lipid content in some micro algae strains could be increased by various cultivation conditions (Illman *et al.*,2000), under stress conditions a lipid production of 30%-60% of dry cell weight has been reported. Among all the factors, nitrogen is known to have the strong influence on metabolism of lipids and fatty acids in various microalgae. In addition, nitrogen is easy to manipulate and is less expensive when compared to other factors. So it is critical to enhancing the lipid productivity for bio-fuel production (Takagi *et al.*,2000). Since fatty acid methyl esters originating from vegetable oils and animal fats are known as biodiesel, from the energetic point of view, lipids are the most desirable components of microalgae cells (Sostaric *et al.*,2009). In addition the organic matter produced by photosynthetic microalgae can be transformed into a wide range of valuable products, such as biodiesel, food additives, health-care products etc (Pulz and Gross, 2004). Biodiesel fuel is becoming more promising as it is from non toxic, biodegradable and renewable resources and its use leads to a decrease in the emission of harmful air pollutants. (Gouveia and Oliveira, 2009).

Biodiesel oil has been mostly produced from oleaginous crops, eg- rapeseed, soybean, sunflower, palm etc. To satisfy the existing demand a large area of land is required for cultivating these energy crops, it may also be necessary to displace 50% of the total area currently used in the United States for food crops (Liu *et al.*,2008). In contrast only 6% of the US cropping area would be sufficient for producing algal biomass with 30% lipid content of dry cell weight, which could completely supply the needed transport fuels (Chisti, 2007). Therefore, microalgae could be a promising alternative feedstock for the next generation of bio-fuels, because they have a relatively high lipid content, high growth rate via cultivation on non-arable land and with non-potable water, and there are no seasonal culture limitations as they can be harvested daily (Gouveia and Oliveira, 2009). To enhance the economic feasibility of using algal oil for biodiesel production, the micro algal biomass productivity, lipid cell content, and overall lipid productivity are the three key parameters that need to be improved. The ideal process is that the microalgae are able to produce lipid at the highest productivity and with the highest lipid cell content. Algal biomass contains on average 50% of carbon in its dry matter. The carbon comes from carbon dioxide necessary for algal growth (Sanchez

Miron *et al.*, 2003). To produce 100 mg of biomass, algae need approximately 183 mg of CO_2 (Frac *et al.*,2010).High lipid contents are usually produced under environmental stress, typically nutrient limitation, which is often associated with relatively low biomass productivity and, therefore, low overall lipid productivity (Li *et al.*, 2008). The lipid content of microalgae could be increased by various cultivation strategies, such as nitrogen depletion (Li *et al.*, 2008), phosphate limitation (Reitan *et al.*, 1994), high salinity (Rao *et al.*, 2007), and high iron concentration (Liu *et al.*, 2008). In the present study, we have used micro algal isolates, *Scenedesmus dimorphus* and *Scenedesmus quadricauda*, with urea as nitrogen source and to induce them to produce lipids suitable for making biodiesel. Strategies leading to optimization of utilizing urea and lipid content/lipid productivity by microalgae were explored. In agriculture, urea is used as a nitrogenous fertilizer, so presence of urea is very common in agriculture wastes which ultimately mix with rivers, ponds, lakes etc. A positive influence of urea as nitrogenous source in the growth of *Spirulina* has been reported, among all organic nitrogenous sources, urea gained importance generally in large scale algal cultivation, because the cost of urea is lower than others (Danesi *et al.*,2002; Matsudo *et al.*,2009). Moreover, urea is the best nitrogen source for culturing *Chlorella* (Becker,1994). Therefore, urea is used for culturing the mentioned two microalgae strains in our present work which may provide an innovative alternative to current biofuel-production strategies (Wang *et al.*, 2008). Commercial-scale cultivation of microalgal biomass is a promising method of producing a renewable feedstock for a wide variety of high-value bio-fuels. It includes methane produced by anaerobic digestion of biomass (Spolaore *et al.*, 2006), biodiesel derived from oil (Banerjee *et al.*, 2002; Gavrilescu and Chisti, 2005), biohydrogen and bioethanol produced by photobiologically active microalgae (Fedorov *et al.*, 2005; Kapdan and Kargi, 2006). The present article expresses the potential of microalgae cultivation for biodiesel production and discusses the utilization of recent engineering techniques for enhancement of lipid profiles in important micro algal strains to make the process more economic and practically feasible.

Material and methods

In the present study two microalgae strains namely *Scenedesmus dimorphus* and *Scenedesmus quadricauda*, isolated from North eastern region of India, Assam were employed. The above mentioned strains were explored in terms of growth rate under different concentration of urea and their lipid productivity in semi continuous mode of cultures.

Medium and culture conditions

BG11 culture media was selected and prepared for the growth of microalgae strains (without adding carbon source) (Rippka *et al.*, 1979) . For our experiment nitrate salt from the media was eliminated and urea was added as a sole source of nitrogen. For this purpose, urea in 0.02, 0.04, 0.08, 0.1, 0.2 g/L were freshly weighed and added to each of the flasks. For the study, before inoculation the pH of the each flasks were adjusted to 7.5 with 0.1N HCl and 0.1 N NaOH with the help of L1 120 pH meter, Elico India. About 150 ml of media were distributed to each of the flasks including the blank and all are inoculated with 20ml of inoculums. (cell densities of inoculums; *Scenedesmus dimorphus=2.25X10*^{7} and *Scenedesmus quadricauda*=3.5X10^7). The Optical Density (O.D) of each the flasks were measured at 680nm of wavelength at regular interval of time (24Hrs) with the help of Systronics spectrophotometer- 104. The strains were checked for 11 days growth period. For our experiment fluorescent lamps were used as a source of light and intensity was adjusted to 2500~3500 lux for all the culture flasks and 16 hrs of light and 8 hrs of dark cycles were repeated for growth of all the cultures. The temperature was adjusted to 25.C for all the flasks.

Analytical methods

Direct microscopic cell count by Neubour haemocytometer was performed using microscope (Labomed). Optical densities of microalgae cultures were measured at regular interval of time (24Hrs) in three replicates and average value was recorded by absorbance at 680nm with the help of spectrophotometer (Systronics). At the end of the experiment all the culture flasks were centrifuged and filtered and dry weights of pellets were measured (80°C for 3 hrs) to study the increase in biomass, cell count and lipid content.

Determination of Specific Growth Rate

Specific Growth Rate is a measure of number of generations (the number of doublings) that occur per unit of time in an exponentially growing culture. The exponential (straight line) phase of growth was carefully determined and specific growth rate was obtained using following equation: (Guillard and Ryther,1962)

$$\mu = \ln (N_t/N_o)/T_t - T_o$$

N_t= No of cells at the end of log phase.
N_o= No of cells at the start of log phase
T_t= Final day of log phase
T_o= Starting day of log phase

If T expressed in days from the growth rate (μ) can be converted to division or doublings per day(k) by dividing(μ) by the natural log of 2.(0.6931).

$$K=\mu/0.6931$$

The time required to achieve a doubling of the no of viable cells is termed as doubling time (T_t) which is calculated by the following formula.

$$T_t = 0.6931/\mu$$

Determination of total lipid

Microalgal lipid extraction was done by Bligh and Dyer method. For that microalgal biomass were collected by

centrifuging the cells at 4000Xg for 10 minutes. The cells were washed with distilled water, lyophilized and weighed. The known amount of biomass (100mg) was then homogenized with chloroform: methanol 1:2 at 35°C.Extract was centrifuged for 7 minutes at 10000Xg and supernatant was collected in a separating funnel. The residue was further homogenized with chloroform and again centrifuged (10000Xg) to collect the supernatant. Now 0.9% NaCl solution was added to the filtrate and washed, lower layer of chloroform was separated and treated with anhydrous Na_2SO_4 to remove the traces of water. The lipid content was determined gravimetrically and expressed as dry weight % after evaporating the chloroform. (Bligh and Dyer,1959).

Results

The growth study conducted on both the indigenous algae confirmed that urea can be used as efficient nitrogenous nutrient source which is well utilized by both the algal strains. From the study it is confirmed that *Scenedesmus dimorphus* contains a large amount of intracellular lipids which starts to accumulate when there is stress condition. Biomass as well as lipid content tends to increase when there is gradual depletion of urea in the media. After 11 days of growth the two strains were kept for 4 days under dark period and closed environment and upon microscopic examination it was observed that cells are much larger in size and shape and showed a different morphology. The lipid content was found to be 34% and 31% (Fig. 3) and a maximum biomass productivity of 1.523 mg/L/day and 1.266 mg/L/day was obtained for *Scenedesmus dimorphus* and *Scenedesmus quadricauda* respectively when they were grown at a urea concentration of 0.1 g/L.

Figure 1: Growth response of *Scenedesmus dimorphus* (at 680nm*)* for 11 days under different concentration levels of urea

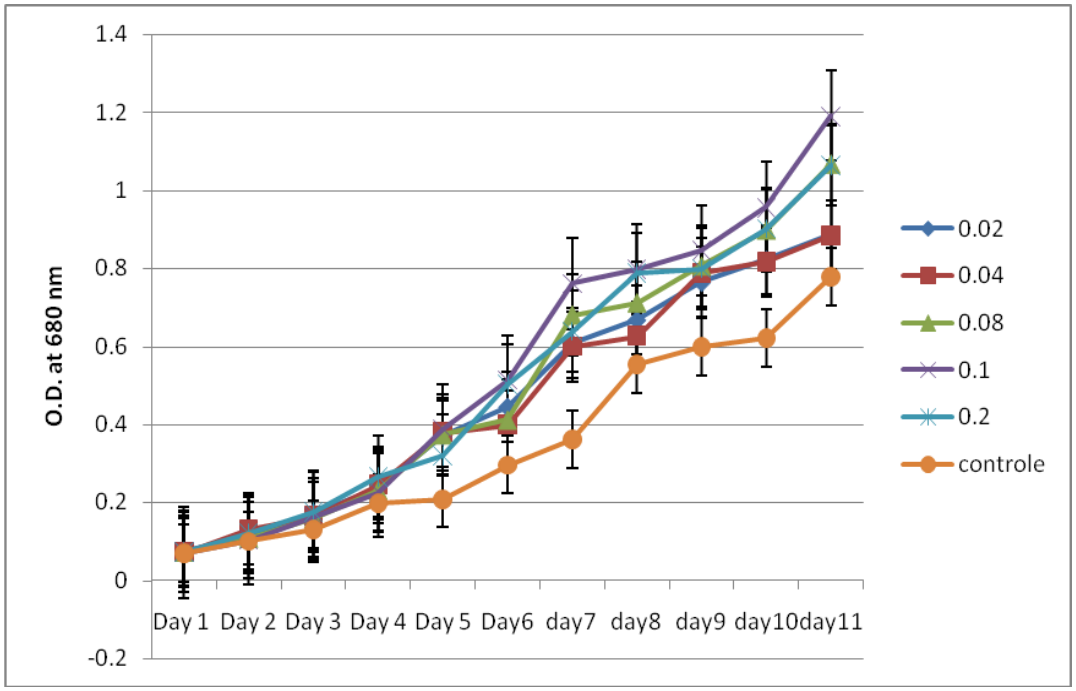

Figure 2: Growth response of *Scenedesmus quadricauda* (at 680nm*)* for 11 days under different concentration levels of urea

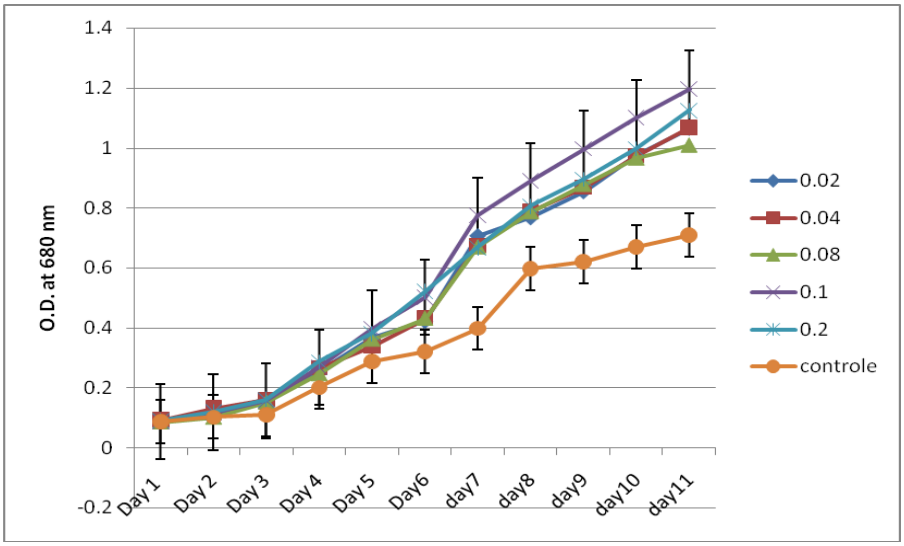

Figure 3: Total lipid content in terms of % dry cell weight in *Scenedesmus dimorphus and Scenedesmus quadricauda* under different concentration levels of urea –

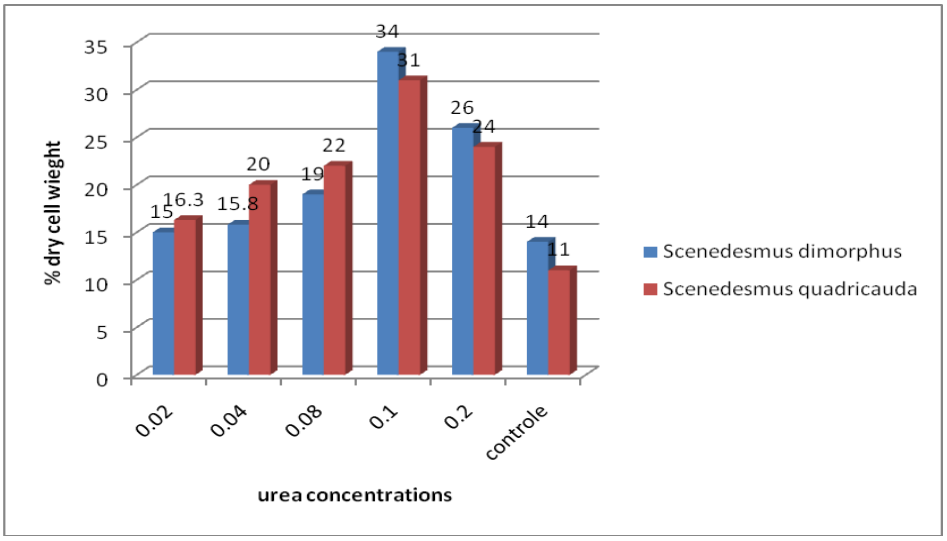

Figure 4: CO2 consumption rate in terms of mg/l/day in case of *Scenedesmus dimorphus Scenedesmus quadricauda* when maximum growth rate was observed under different growth conditions. (Calculated from the biomass productivity according to the following equation: CO2 fixation rate (PCO_2)=1.88×biomass productivity (mg L−1 d−1), which is derived from the typical molecular formula of microalgal biomass, CO0.48H1.83N0.11P0.01 (Chisti, 2007)).

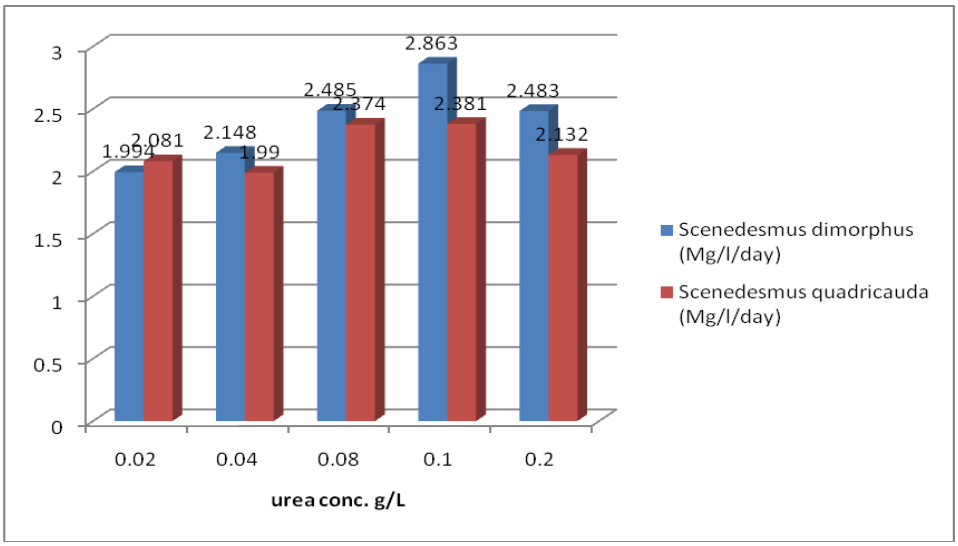

Table-2 depicts for increase in cell number per day (cells/day) and increase in biomass per day (mg/ml/day) of the two strains in different concentration of urea. Specific growth rate and doubling/ day was recorded to be 0.54 and 0.779 for *S. dimorphus* and 0.392 and 0.562 for *S. quadricauda* at 0.1 g/L urea concentration for both the strains (Table-1). Comparison of the CO_2 fixation performance of *Scenedesmus dimorphus* and *Scenedesmus quadricauda* strains under different concentration of urea were also given in the Table 3. Surprisingly when urea concentration was increased above 0.1 g/L the biomass as well as lipid content of both the strains showed a significant decrease. So it is well understood that urea sufficient and urea deficient both the conditions show different cell growth behavior. A high amount of urea does not show increased biomass and cell count of the two strains

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Table 1: Table for specific growth, doubling per day and doubling time of *Scenedesmus dimorphus and Scenedesmus quadricauda* under different concentration of urea.

Parameter	*Scenedesmus dimorphus*			*Scenedesmus quadricauda*		
Urea g/L	Specific growth	Doubling/day	Doubling time	Specific growth	Doubling/day	Doubling time
0.02	0.452	0.652	1.53	0.366	0.527	1.89
0.04	0.436	0.629	1.59	0.242	0.349	2.86
0.08	0.479	0.691	1.44	0.383	0.553	1.81
0.1	0.54	0.779	1.28	0.392	0.562	1.77
0.2	0.188	0.271	3.68	0.282	0.407	2.46
Control	0.141	0.202	4.91	0.358	0.516	1.93

Table 2: Table for increase in cell number per day (cells/day) and increase in biomass per day (mg/ml/day) of *Scenedesmus dimorphus and Scenedesmus quadricauda*.

Parameter	*Scenedesmus dimorphus*		*Scenedesmus quadricauda*	
Urea g/L	Increase in cells/ml/day($X10^4$)	Biomass mg/ml/day	Increase in cells/day	Biomass mg/ml/day
0.02	1.05	1.061	1.12	1.107
0.04	1.15	1.143	1.06	1.059
0.08	1.33	1.323	1.19	1.262
0.1	1.61	1.523	1.31	1.266
0.2	1.23	1.321	1.11	1.134
Control	0.998	1.012	0.733	0.739

Table 3 : Comparison of the CO_2 fixation performance of *Scenedesmus dimorphus* and *Scenedesmus quadricauda* strains under different concentration of urea.

CO_2 consumption rate.		
Urea concentration g/L	*Scenedesmus dimorphus* (Mg/l/day)	*Scenedesmus quadricauda* (Mg/l/day)
0.02	1.994	2.081
0.04	2.148	1.99
0.08	2.485	2.374
0.1	2.863	2.381
0.2	2.483	2.132
Control	1.902	1.389

Calculated from the biomass productivity according to the following equation: CO2 fixation rate (PCO_2)=1.88×biomass productivity (mg L−1 d−1), which is derived from the typical molecular formula of microalgal biomass, CO0.48H1.83N0.11P0.01 (Chisti, 2007).

The results indicated that a concentration of 0.1 g/L urea for both the strains is optimum nutrient condition as nitrogenous source and for lipid productivity, as urea is a very cheap nitrogenous nutrient, so it can be exploited for mass culture of both the indigenous microalgal strains to promote commercial production of lipid for alternative fuel generation. However the only problem which showed its existence while cultivating *Scenedesmus* strains were both the strains were heavy as a result they tends to settle down which slower their growth so proper agitation is necessary for both the strains.

Discusssion

From the study it is obvious that the growth behavior and lipid accumulation of microalgae are strongly related to nitrogen concentration in the media. Urea can be used as a very efficient nitrogen source for microalgae cultivation in large scale, the advantage which urea delivers is it is very cheap compared to other nitrogenous nutrients available for microalgae which ultimately make it economically suitable for industrial production of microalgal fuel. Zhila *et al*. (2005) cultured *Botryococcus braunii* in 75% reduced N medium with 1% CO_2 at 10:14 light-dark period during the 20 days and observed the growth of the algae and lipid composition. When the culture of 75% reduced N was compared with the control group, it was observed that, the biomass decreased from 6.8 to 2.9% and the lipid ratio increased to 21%. In the other study, *Neochloris oleoabundas* was cultured in photobioreactor system in N deficient medium and the biomass and lipid levels were examined. While 16.5 g m -2/day biomass and 23% lipid were recorded in the control group, low biomass and 37% of lipid were obtained in the culture to which applied N was deficient (Pruvost *et a*l., 2009). Two green algae (*Chlorella vulgaris* and *Scenedesmus obliquus*) and four blue green algae (*Anacystis nidulans, Microcystis aeruginosa, Oscillatoria rubescens and Spirulina platensis*) were grown in 81 batch cultures at different N levels. In all the algae increasing N levels led to an increase in the biomass (from 8 to 450 mgL-1), in protein content (from 8 to 54%) and in chlorophyll. At low N levels, the green algae contained a high percentage of total lipids (45% of the biomass) (Piorreck et al., 1984). Reitan et al. (1994) cultured the microalgae *Phaeodactylum tricornutum, Chaetoceros s*p. (Bacillariophyceae), *Isochrysis galbana* , *Nannochloris atomus* (Chlorophyceae), *Tetraselmis sp.* (Prasinophyceae) and *Gymnodinum sp.* (Dinophyceae) at different lacking nitrogen media and observed the increase of lipid, whereas Widjaja (2009) cultured *C. vulgaris* in the nitrogen deficient medium for the periods of 7 and 17 days. At the end of the 17 days culture period, the total lipid was found to be higher. So the N-source in the media greatly effects the lipid composition of microalgae. In our study various levels of urea concentration as N-source were investigated to analyze biomass production and lipid content. The maximum lipid production of 34% in terms of dry cell weight was obtained when urea level was at 0.1 g/L *S. dimorphus* strain. The result implies that appropriate amount of urea feeding leads to a good production of lipid in case of microalgae. After eleventh day of culture when cells were kept at dark and in a closed environment, it triggers lipid production as urea concentration depletes quickly and cells are in a starved condition which actually induces lipid production. It is also noticed that increased urea concentration can effectively enhances biomass production but the increased biomass does not always imply to an increased lipid content as increased urea levels may enhances biomass production but cells may have a low lipid content. So the best strategy is to growing cells under an optimum urea concentration for a specific period of time (11 days in case of our experiment) to obtain a good amount of biomass and than there should be an introduction of a urea starving condition which ultimately lead to synthesis of high amount of lipid (where cells will be at stationary phase).

Acknowledgement

Authors are thankful to Gauhati University and Deptt. of Biotechnology, Gauhati University for providing facilities to carry out the research work. All the financial assistance for the research work were provided by Department of Science and Technology (DST) Govt. of India.

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