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Effect of Nitrogen on Growth and Lipid Content of *Chlorella pyrenoidosa*

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Abstract: Problem statement: In today's scenario, microalgae are widely recognized as a promising source for biofuel production as a renewable source of energy. They are exceedingly rich in oil, which can be converted to biofuel. This paper deals with one of the method to enhance the lipid content in microalgae. **Approach:** Microalga, *Chlorella pyrenoidosa*, was grown autotrophically in batch culture and the effect of different concentrations of nitrogen source (0-0.4 g L^{-1} KNO_3) on growth and lipid content was studied. **Results:** As the nitrate concentration in the medium decreased, biomass production also decreased but the lipid content increased. Moreover, at the same concentration of nitrate source, lipid tends to accumulate more in stationary phase in comparison to exponential phase. Highest lipid accumulation of 26% was recorded in the culture with 0.05 g L^{-1} KNO_3, which is one-fourth of basal nitrogen source concentration. **Conclusion:** The present study suggested that nitrogen starvation is the effective approach to enhance lipid for biofuel production.

Key words: Microalgae, lipid content, nitrogen source, KNO_3, biofuel, comparison to exponential, grown autotrophically, batch culture, Triacylglycerols (TAGs)

INTRODUCTION

Biodiesel is an alternative to standard diesel. It is usually made from bio-oils through a process called transesterification. There can be various sources of oil like waste cooking oil and vegetable oil of oil plants like soyabean, sunflower, jatropha, palm oil (Khalid and Khalid, 2011). Studies of Awang and May (2007) have shown that palm oil can be used as enviornmental friendly alternative fuel source. It has also been proved that biodiesel produced from waste cooking oil has lesser sulphur content than existing diesel fuel. Also, the accumulation of the CO and HC emissions decreased significantly (Najafi *et al*., 2007). These first generation fuels are not feasible for large scale production as they compete for land and water used for agricultural practices (Takeshita, 2011). To reduce the cost of biodiesel production, low cost waste materials need to be used as feedstocks which will produce enviornment friendly fuel as well as reduce pollution potential of the wastes (Ghaly *et al*., 2010).

Algae seems to be a promising source for biodiesel production. They are photosynthetic microorganisms which convert sunlight, water and CO_2 to sugars, from which macromolecules such as lipids and Triacylglycerols (TAGs) can be obtained (Singh and Gu, 2010). These TAGs are the sustainable feedstock for biodiesel production. Microalgae are far more efficient and can be grown in non-arable land, utilize waste water and do not compete with food crops for land and water (Chisti, 2007). They are renewable and environment friendly as they have the ability to fix CO_2 and so can also be an interesting method of reducing greenhouse gases (Mata *et al*., 2010). Common species of macroalgae like Oedogonium and spirogyra have also been used for the production of biodiesel (Shariff Hossain *et al*., 2008). But, most of the work has been concentrated on microalgae because of their high lipid content.

The oil productivity of microalgae is more than best producing oil crops (Converti *et al*., 2009). The oil or lipid extraction from algae has already been studied and tested using different solvents like hexane/isopropanol (Gunnlaugsdottir and Ackman, 1993), hexane/ethanol (Fajardo *et al*., 2007), chloroform/methanol (Bligh and Dyer, 1959). The study of Long and Abdelkader (2011) has shown that chloroform/methanol protocol give higher yield of lipids from microalgae *Nannochloropsis sp.* than a hexane/isopropanol mixture.

For commercial production of biodiesel from microalgae, research and development is needed in several aspects. Suitable strains need to be selected and optimized for biomass production and lipid profile

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(Pruvost *et al*., 2011). Some of the species of microalgae already possess high oil content and they can be manipulated to produce more oil (Gao *et al*., 2010). A number of factors have been shown to influence the lipid content of algae, such as nitrogen deficiency (Yeesang and Cheirsilp, 2011; Gouveia and Oliveira, 2009), phosphate limitation (Gouveia and Oliveira, 2009), salt stress (Hu and Gao, 2005), temperature fluctuation (Zhu *et al*., 1997). Light intensity and iron content of the medium also affect algal growth along with lipid content (Tang *et al*., 2010; Yeesang and Cheirsilp, 2011).

Several studies have shown that lipid tends to accumulate in nitrogen deficient conditions (Pruvost *et al*., 2011; Gouveia and Oliveira, 2009). In general, there is an inverse relationship between lipid content and nitrate concentration (Gouveia and Oliveira, 2009).

The present study was conducted to investigate the growth response and lipid content of a freshwater green alga, *Chlorella pyrenoidosa*, by varying the concentrations of nitrate (KNO3) in the growth medium. Lipid content in both exponential and stationary phase was estimated to study the effect of nitrogen stress in this alga.

MATERIALS AND METHODS

Organism and growth conditions: The culture of green alga *Chlorella pyrenoidosa* was provided by National Chemical Laboratory of Pune, India. The cultures were grown in 1000 ml Erlenmeyer flasks with 500 mL medium. The medium used for cultivation was Fogg's medium (Fogg, 1949; Fogg, 1973). The cultures were performed in a temperature controlled incubator at 25°C providing 24 h fluorescent illumination (40 watt, white tube light). The cultures were hand shaken two to three times daily to avoid sticking. All the glassware and media were always sterilized prior to innoculation. This was referred to as control culture. All the experiments were carried out in triplicates.

Cell growth analysis: Optical density measurement at 660 nm was used to monitor cell growth by UV/visible spectrophotometer (Shimadzu UV-1650). All cultures were initiated with an O.D. of about 0.1. Cells were concentrated by centrifugation, washed with de-ionized water and dried (60°C) to determine dry weight (expressed as g/l). Based on the calibration curve of O.D. and dry biomass concentration, 1O.D. corresponds to 0.636 g L^{-1}. So, biomass concentration was calculated by regression equation: $y = 0.636\times(R^2 = 0.9933, p<0.05)$, where y (g/L) is the dry cell weight, x is the absorbance of suspension at 660 nm.

Lipid extraction: Extraction of lipid was done following the protocol of Bligh and Dyer (1959). 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 recentrifuged. The pellet was, then, subjected to wet weight estimation and then 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. After that, 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 (W_1). Evaporation was carried out in hot air oven at 80°C for 50 min. The weight of the vial was again recorded (W_2). Lipid content was calculated by subtracting W_1 from W_2 and was expressed as % dry cell weight.

Nitrogen starvation: The alga was studied for growth and lipid content in different concentrations of nitrate. The original nitrogen source concentration in the medium was 0.2 g L^{-1} KNO_3. The experiment was performed in 0, ¼, 1/2 and double of the original nitrogen source concentration. The effect of initial nitrogen concentration on the microalgal growth and lipid content was investigated. Lipid content was estimated after 12 days (exponential phase) and 24 days (stationary phase) of inoculation.

Statistical analysis: Each measurement was done in triplicate and the mean and standard deviation of the experimental results was calculated using MS-Excel.

RESULTS

Figure 1 show the time course biomass profile of *Chlorella pyrenoidosa* grown autotrophically in Fogg's medium with different concentrations of KNO_3 in the medium. The original nitrogen source concentration in the medium was 0.2 g L^{-1} KNO_3. Different sets of nitrogen concentrations - 0, ½, ¼ and double of control was used to investigate the effect of nitrogen on growth and lipid content of the organism. Growth of alga increased after a short lag phase of 2 days followed by a logarithmic phase and attained stationary phase at about 24 days. As evident from the graph (Fig. 1), by increasing the KNO_3 concentration, growth also increased.

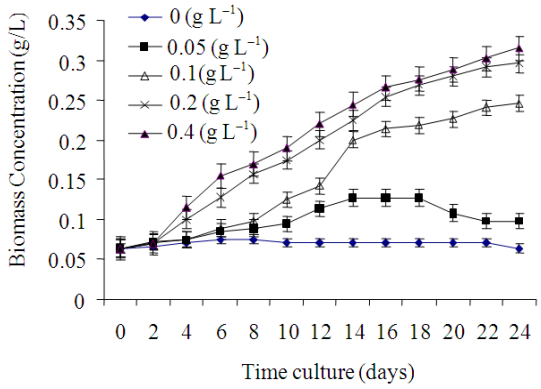

Fig. 1: Growth curve of Chlorella pyrenoidosa grown on Fogg's medium with different initial KNO3 concentration (0-0.4 g L^{-1})

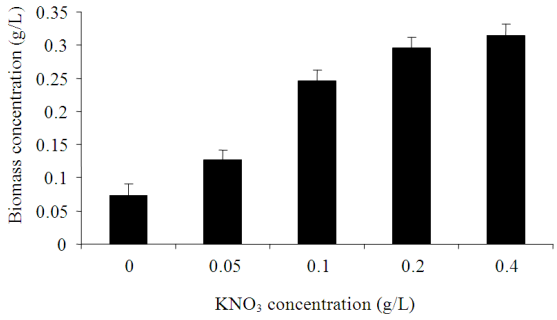

Fig. 2: Highest biomass production of Chlorella pyrenoidosa grown on Fogg's medium with different initial KNO3 concentration (0-0.4 g L^{-1})

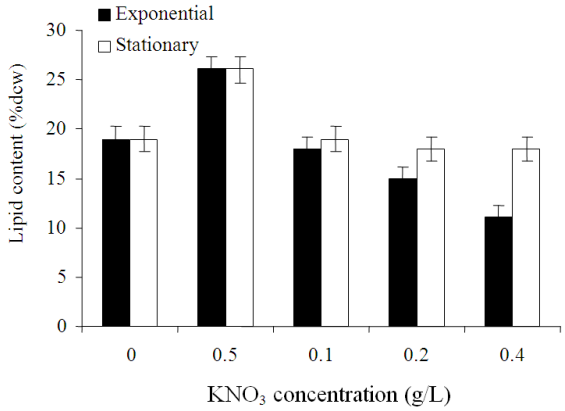

Fig. 3: Comparison of lipid content of Chlorella pyrenoidosa grown on Fogg's medium with different initial KNO3 concentration (0-0.4 g L^{-1}) in exponential and stationary phase

Table 1: Effect of nitrogen concentration on biomass production and lipid content of *Chlorella pyrenoidosa* in exponential and stationary phase

Concentration of KNO_3 (g/L)	Max. biomass production (g/L)	Lipid content exponential phase	(% dcw) stationary phase
0	0.075	19	19
0.05	0.127	26	26
0.1	0.246	18	19
0.2	0.296	15	18
0.4	0.315	11	18

In the absence of a nitrogen source (0g L^{-1} KNO_3), no growth was observed and the cells appeared bleached. At day 24, maximum biomass concentration of 0.315 g L^{-1} was recorded in the culture with double the concentration of nitrate (0.4g L^{-1} KNO_3). Dry matter of 0.296, 0.246 and 0.127g L^{-1} was observed in cultures with 0.2g L^{-1} KNO_3 (control), 0.1g L KNO_3(1/2 of control) and 0.05 g L^{-1} KNO_3 (1/4th of control) respectively (Fig. 2).

Figure 3 and Table 1 shows the lipid content of Chlorella pyrenoidosa in different concentrations of nitrate in both exponential and stationary phase. An increasing trend is observed in lipid content as the concentration of nitrate was decreased. Also, at the same concentrations of nitrate, stationary phase cultures showed higher lipid accumulation in comparison to that of exponential phase. When the concentration of nitrate was doubled (0.4g L^{-1} KNO_3), the lipid content in the exponential phase was 11% dry cell weight as opposed to control (15%). However, in stationary phase, 18% was recorded in both the cultures.

Moreover, when the nitrogen source was decreased from 0.2 g L^{-1} KNO_3 to 0.1g L^{-1} KNO_3 (1/2 of control), the lipid content in exponential phase increased to 18% from 15%. Stationary phase cultures did not show much difference (19%) in lipid content when grown in 0.1g L^{-1} KNO_3.

A sharp rise in lipid accumulation of 26% (both in exponential and stationary phase) was recorded when the cultures were grown in initial nitrogen concentration of 0.05g L^{-1} KNO_3 (1/4th of the original concentration).

Cultures transferred to nitogen-free medium (0g L^{-1} KNO_3) with no initial nitrogen source led to enhancement of lipid content of 4% (of control) attaining a value of 19% in both exponential and stationary phase.

DISCUSSION

For economical production of biofuel from microalgae, biomass as well as lipid content play an important role. In the present study, the results mentioned above indicate that high concentration of nitrogen source supported the biomass concentration in contrast to the lipid content (Table 1). The alga,

Chlorella pyrenoidosa, cannot grow without a nitrogen source and its growth is directly proportional to the concentration of nitrate in the medium (Fig. 1). As nitrate source is increased in the medium, enhancement in biomass concentration was recorded (Fig. 2). This result is in accordance with that of Chittra and Benjamas (2011). They have reported loss of biomass when green alga, *Botrycoccus spp.* was exposed to nitrogen deficient conditions (Yeesang and Cheirsilp, 2011). Mandal and Mallick (2009) have also reported decreased growth pattern in *Scenedesmus obliquus*, under nitrogen deficient conditions (Gouveia and Oliveira, 2009). Decrease in algal biomass concentration in low nitrate concentration was also seen by Hanhua and Gao in *Nannochloropsis* sp. Hu and Gao, 2005).

The present study has also proved that lipid content rises as nitrate concentration declines in the medium (Fig. 3). According to Yeesang and Cheirsilp (2011), under nitrogen deficient conditions, algal cells accumulate carbon metabolites as lipids (Yeesang and Cheirsilp, 2011). It has earlier been reported that under nitrogen starvation conditions, nitrogen containing macromolecules and carbon reserve compounds like carbohydrates and fats are accumulated (Banerjee *et al*., 2002; Dayananda *et al*., 2006). Our study also shows that lipid tends to accumulate more in stationary phase than in exponential phase (Fig. 3 and Table 1). Under growth limiting conditions, e.g., when cells have reached stationary growth phase, more carbon is incorporated into carbohydrates and lipids (Zhu *et al*., 1997).

Figure 3 shows that when cells are transferred to nitrogen rich medium (double of control), lipid content was decreased by 4% as compared to control. However, in stationary phase, lipid content was same (18%) as control. This might be because as the algae reaches stationary phase, it has used up its nitrogen reserves for growth and now, it starts accumulating lipid for its survival. This is in parity with Zhu *et al*. (1997). On the contrary, when the initial concentration of nitrogen source was half of its original concentration (0.1g L^{-1} KNO_3), lipid content was higher by 3% as opposed to control. It seems that the alga was already growing on nitrogen deficient conditions from the beginning and so lipid accumulation was, nearly, same in both exponential (18%) and stationary phase (19%). The optimum initial nitrate concentration seems to be 0.05 g L^{-1} KNO_3, i.e., $1/4^{th}$ of the original nitrate concentration. At this concentration, a sharp enhancement in lipid accumulation at logarithmic phase was recorded (26%) which is 11% higher to that of control (15%). This result is in agreement with earlier findings of Yeh and Chang (2011) *Chlorella vulgaris*, where lipid content increased from 20.9-55.9% when concentration of nitrogen source was decreased from 1.25- 0.313 g L^{-1} (Yeh and Chang, 2011). Hanhua and Gao has reported a four-fold increase in lipid content in *Nannochloropsis* spp. when subjected to low nitrogen source conditions (Hu and Gao, 2005). Nitrate starvation has shown to trigger lipid accumulation in freshwater algae, *Chlorella vulgaris* and *Neochloris oleoabundans.* The highest total lipid content was reported in *Neochloris oleoabundans* (25-37% of DW), while the highest TAG content was found in *C.vulgaris* (11-14% of DW) (Pruvost *et al*., 2011). This result is also in parity with that of where *Nannochloropsis oculata* showed a gradual decrease in the growth rate accompanied by almost a duplication of lipid content.

Transferring cells to nitrogen-free medium, with no nitrogen source also led to an increase in lipid content of 4% against control after 12 days. Yasemin *et al*. (2011) have recorded nearly three-fold increase in lipid in *Chlorella vulgaris*, when grown in nitrogen-free medium (Mutlu *et al*., 2011). Enhancement in lipid production up to 43% (dry cell weight) has been obtained when cultures of *Scenedesmus obliquus* were transferred to media deficient in nitrate for 7 days (Gouveia and Oliveira, 2009).

Recently, for biodiesel production, researchers are working on growing *Chlorella sp.* heterotrophically by providing an organic carbon source. Miaoa and Wu (2006) cultured *Chlorella protothecoides* autotrophically and found 14.57% lipid. In the same study, they reported lipid of 55.20% in heterotrophic growth (Miaoa and Wu, 2006). However, heterotrophic microalgal growth is difficult to adopt for commercial use as it is quite expensive (O'Grady and Morgan, 2010).

CONCLUSION

The present study suggests, the most effective approach to enhance lipid in *Chlorella pyrenoidosa* is to grow it autotrophically in growth medium with initial concentration of 0.05 g L^{-1} KNO_3. This gives an 11% hike in lipid content over control.

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