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Estimation of Lipid Accumulation in *Chlorella pyrenoidosa* culturing in Different Concentrations of KNO_3

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Abstract - The present study deals with growing microalgae Chlorella pyrenoidosa to assess its biofuel potential. The microalga was grown in Fogg's media with different concentration of nitrate source. And an attempt has been made to increase lipid content in algae in nitrogen stress condition. Chlorella pyrenoidosa was isolated from sewage water by step dilution method. Different concentration of KNO_3 (0-0.4g/L) was used in Fogg's media. Eventually biomass production was decreased with decreasing concentration of nitrate in media but lipid content increased. The result showed that lipid accumulation is higher in stationary phase than in exponential phase at same concentration of nitrate. In culture media with 0.05g/L of KNO_3 maximum lipid content of 28% was recorded. The experimental study concluded that nitrogen starvation is a promising method to enhance lipid productivity in microalgae and it can be a big tool in biofuel production project.
Keywords- Biomass, Nitrogen starvation, TAG, Exponential phase, Dry Cell Weight, Fogg's media, Lipids.

I. INTRODUCTION

Biomass is organic matter derived from living or recently living organisms. Biomass is a term used for all living matter on Earth. Biomass energy is a source of renewable energy developed at faster rate in form of aquaculture and agriculture. Biomass energy is usually stored in wood, land crops and algae for conversion into biogas & biofuel. Biomass energy not only solves the problem of energy shortage but is also beneficial for environment as it does not cause pollution like that of fossil fuel like coal, petroleum etc. However the use of land plants poses the problem of sustainability that is need of land to grow crops for an increasing population. Biofuels pose an attractive option and a lot of research has been dedicated to this area. The first generation biofuels are fuels made from sugar, starch, vegetable oil etc. Second generation biofuels use biomass or liquid technology including cellulosic biofuels. Third Generation Biofuels are also called "Oilgae" or biofuel from algae. Algae are high yield feed stocks with low input, to produce biofuels. On basis of laboratory work, it has been claimed that these green micro-plants have ability to produce 30 times more energy per acre than land crops [1]. One of many benefits of biofuels is that they are bio-degradable and relatively harmless to our environment if accidently spilled. Algae have highest biofuel potential. Algae are photosynthetic in nature, fixes CO_2 in presence of sun light & manufacture own food, assimilating it as glucose. Some microalgae species are "oil rich" energy crops producing 10-100 times more biomass energy than other traditional crops. Many species of micro algae can be induced to produce good quantities of lipids, which contribute to more oil yield. Normally lipid content of micro-algae varies between 1-70% but under control culture conditions it can be enhanced to 92% of dry weight. For microalgae culture to produce 'Oilgae' composition of fatty acids plays a significant role on characteristics of biodiesel produced. Cultivation of microalgae at large scale has a 10-20 times more productive value for biofuel production as compared to other biofuel crops. Algae cultivation for production of sustainable biofuels is mainly effected by physical and biological aspects which make up the whole system of algal biofuel production. Till now thousands of algal species have been screened which are capable to produce high lipid content. The most important factors which affect the biomass production and lipid productivity in algae are light, temperature, pH and nutritive media. In microalgae triacylglycerides (TAG) act as storage lipids. Mostly TAG made of little unsaturated fatty acids and mostly saturated fatty acids and by transesterification process these fatty acids can be converted in biofuel [7]. Many species of microalgae already possess high oil content and they can be manipulated to produce more oil [6]. However, a no. of microalgal species have been discovered which have capability for accumulation of high levels of long chain of poly unsaturated fatty acids (PUFA) as TAGs. A number of factors have been shown which influence the lipid content of algae, such as temperature fluctuation [3], nitrogen deficiency([8], [9]), salt stress ([3], [4]) and

phosphate limitation [11]. Iron content and light intensity of the medium also affect algal growth along with lipid content [2]. Several studies have shown that in nitrogen deficient conditions, the lipid tends to accumulate in cells. There is an inverse relationship between lipid content and nitrate concentration in media [1]. It has been observed that under certain modified culture conditions such as nitrogen starvation change in pH, by lowering or increasing temp. leads to increase production of PUFA rich TAG which constitute 10-20% of their dry cell weight (DCW) and an increased accumulation of neutral lipids (25-60% DCW) in form of TAG which help microalgae to endure these adverse conditions.

Based on the literature reviewed, it is clear that nitrogen starvation treatment is most studied and widely applied in almost all the microalgae species that can be considered best for the commercial production of biodiesel. The present study was focused on changes in biomass production and lipid content of a green algae *Chlorella pyrenoidosa* grown in different concentration of nitrogen source. Lipid production in exponential phase and stationary phase in nitrogen stress conditions also had been studied.

II. MATERIALS & METHODS

A. Sample collection

Samples of waste sewage water were collected from Kholriwal Waste Water Treatment Plant, Jalandhar. The algae were isolated from sewage water by dilution method. Each serial dilution was poured by streaking on agar solidified Fogg's media. Compound microscope was used for observing different types of algae. *Chlorella pyrenoidosa* was identified and selected for further study.

B. Culture of algae

C.pyrensidosa was cultured in 500mL Erlenmeyer flasks with 250mL of Fogg's medium. [5]. The cultures were grown in incubator under controlled temperature at 25^0C, providing 16:8 light/dark conditions. The culture flasks were shaken manually with hand three to four times daily to prevent sticking with the glass walls. Before starting inoculation the media and all glass were sterilized. This was taken as control culture.

C. Dry Cell Weight analysis

Biomass content was determined by taking optical densities of the culture samples at 660nm. Dry weight was calculated by taking 3ml of culture sample in centrifuge tube, which was centrifugated at 2500 rpm for/10min. After discarding supernatant, the centrifuge tube with pellet was dried in oven at temp 45^0C for 2hours to get constant weight. To get DC's of algae, weight of centrifuge tube at end was subtracted from weight at beginning. DCW was measured only 0, 2, 5, 9, and 14). The conversion factor was calculated by plotting Dry Cell Weight (DCW) versus OD at 640nm.

The linear regression equation was obtained as

$$Y = 0.6521 \times R^2 = 0.98232, P < 0.05$$

Where Y is DCW of microalgae Cells,

X = O.D at 640nm.

D. Nitrogen starvation treatment

C.pyrenoidosa was used to study effect of different conc. of KNO_3 on cell growth and lipid content. The original concentration of KNO_3 is medium was 0.2g/L of KNO_3. The experiment was conducted in 25%, 50%, 100%, and 200% of original concentration. The effect of different conc. of nitrate on biomass and lipid productivity was observed. Lipid productivity was estimated on 9 day (Exponential phase) and 20^{th} day (Stationary phase).

E. Lipid content analysis

Lipid content analysis of algae was done using method as proposed by Bligh and Dyer (1959). An algal suspension sample was centrifugated for 10 minutes at 3800 rpm. A concentrated algae paste was obtained and this pellet was weighed for wet weight estimation. This paste was dried at temp 80^0C for 2hours.Then for 1g of algal biomass 1mL methanol and of 2mL of chloroform was added. This suspension was left as such for 24 hrs. at 18^oC. Then 1 mL of chloroform was again added and this solution was mixed for 1 min on vortex. Than 2 mL of water was added and agitated again for 2 min. Then layers were separated by centrifugation at 2000 rpm for 10min. With help of glass syringe lower layer with lipids was extracted and transferred to pre weighed vial (W_1). Using water bath solvent was evaporated and vial was again weighed (W_2). Lipid content was calculated as W_1 - W_2 and expressed as % dry cell weight.

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III. RESULTS AND DISCUSSION

The microalgae were isolated from sewage water by serial dilution method. *Chlorella pyrenoidosa* was identified by microscopic examination. The culture was grown in Fogg's media autotrophically and with different concentration of KNO_3 in media. Different concentration used was 0%, 25%, 50%, 100% and 200% of control.

Fig. 1 Biomass concentration of Chlorella sp. in different concentrations of KNO_3. On 12th day (exponential phase)

Fig. 1 shows biomass concentration in different concentration of KNO_3. With increasing conc. of KNO_3, the growth also increased. In nitrate free medium, no growth was observed and cells become bleached. The biomass production profile of *Chlorella* with due time course has been shown in Fig 2. After lag phase of 3 days, the growth increased in logarithmic phase (10 days) and stationary phase was attained at about 20 days. In culture with double concentration of KNO_3 maximum biomass production of 0.325g/L was observed. Dry matter of 0.307g/L, 0.263g/L and 0.129g/L was recorded in culture with 0.2g/L (100% control), 0.1g/L (50% of control) and 0.05g/L (25% control) respectively.

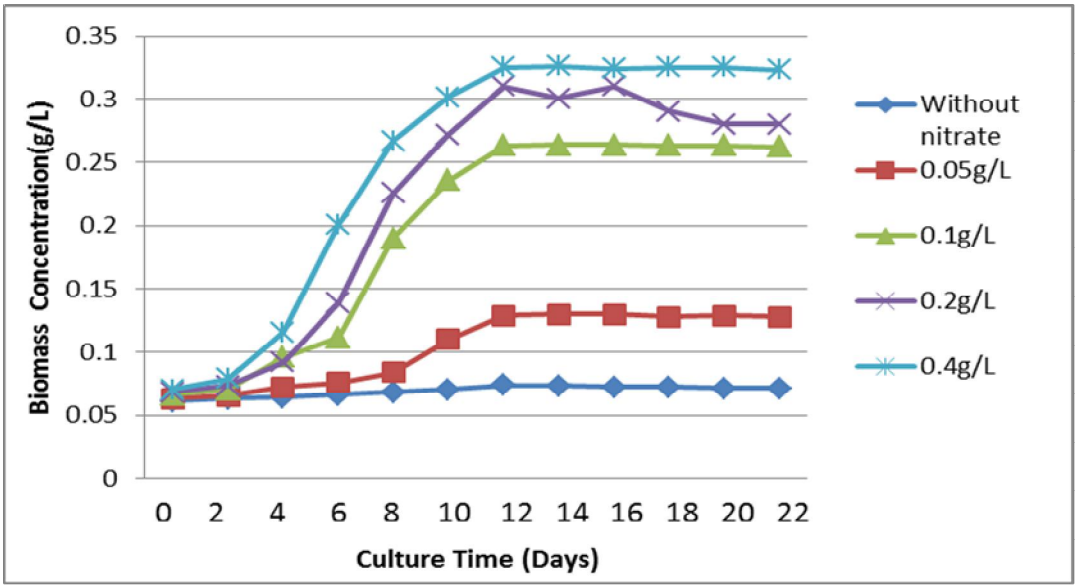

Fig. 2 Growth curve of Chlorella pyrenoidosa grown on Fogg's medium with different concentrations of KNO_3 (0-0.4 g/ L)

Figure 3 shows the lipid productivity in *Chlorella* sp. in varying concentration of KNO_3 in stationary and exponential phase. As concentration of KNO_3 was decreased lipid productivity was increased.

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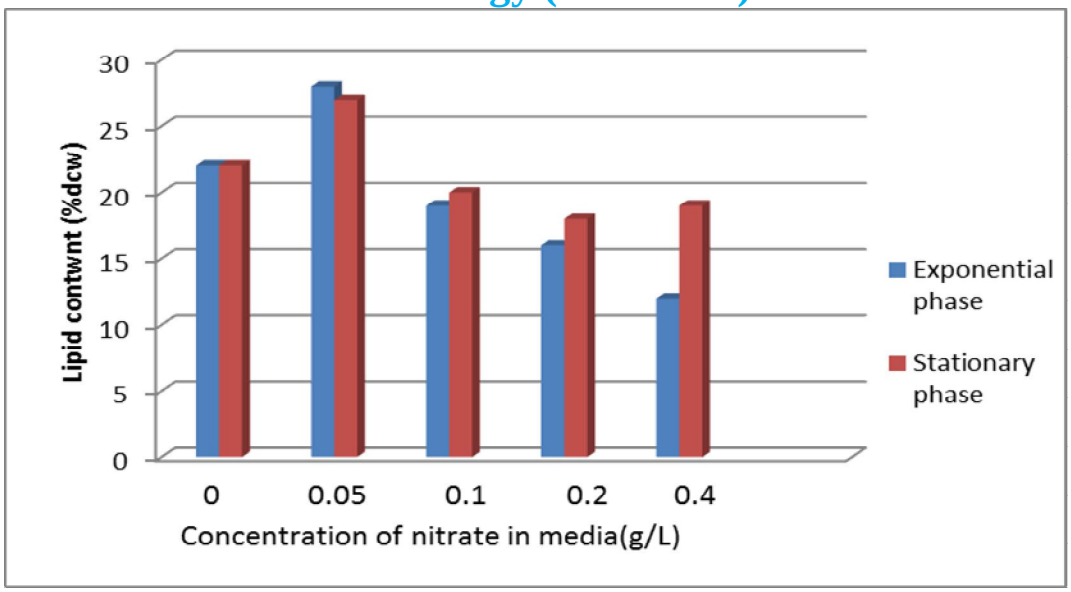

Fig.3 Comparison of lipid content of Chlorella in different concentrations of KNO_3 both in exponential and stationary phase.

The culture showed more lipid accumulation in stationary phase than the exponential phase even at the same concentration of KNO_3. In double the concentration of KNO_3 i.e. 0.4g/L. the lipid content was 12% of DCW in exponential phase as opposed to 19% in stationary phase has been recorded. In culture with 50% of nitrogen source (0.1g/L of KNO_3) during exponential phase lipid content increased to 19% from 16% in control but a little difference of lipid content in stationary phase. When the *Chlorella* was grown in $1/4^{th}$ of original conc. (0.05 KNO_3) there was a sharp increment of lipid productivity of 28% (both in stationary and exponential phase). In cultures without nitrogen source lipid content was enhanced by 6% (than to control) and attained a value of 22% (both in stationary & exponential phase).

Chlorella pyrenoidosa requires nitrogen source for its growth. Rate of growth is directly proportionate to nitrate concentration in media. The loss in biomass production has been previously recorded in *Scendesmus obligus*, when it was grown under nitrogen starvation conditions. [13]. Algal cells start accumulating carbon metabolites as lipids under nitrogen starvation conditions [2]. When we cultured *Chlorella pyrenoidosa* in 50% conc. of KNO_3 (i.e. 0.1g/L) lipid content increased by 3% as compared to control. At 25% concentration of KNO_3 (i.e. 0.05g/L) lipid content increased by 12% compared to control. So as the nitrate concentration was decreased lipid accumulation increased in algal cells.

The present study also showed that lipid accumulation is higher in stationary phase as compared to exponential phase (Fig.3) because more carbon incorporates into carbohydrate & in turn lipids. From Fig 3 it is clear that when algal cells are cultured in 200% KNO_3 conc. i.e. (0.4g/L), lipid content was lowered by 4% compared to control in exponential phase. But lipid content was almost same i.e. 19% in stationary phase compared to control, because when algal cells cultured in nitrate rich source, they used up whole NO_3 for growth upto stationary phase and now start lipid accumulation for its survival. But when algae cells are cultured in nitrogen starvation conditions i.e. 0.1g/L lipid content is nearly same in both exponential phase (19%) & stationary phase (20%) because microalgae growing under nitrogen starvation conditions, starts lipid accumulation earlier in exponential phase. So it remains nearly same in both phases.

The final results reveal that optimum nitrate concentration appears to be 25% of control (i.e. 0.05g/L) in culture. There is a sharp increment of lipid content (28%) in exponential phase. This is found to be 12% higher than under control (16%). This result is in parity with early findings of increment in lipid accumulation by 3-4 folds in *Chlorella vulgaris*, when cultured in nitrogen free media [1]. Nearly 43% lipid production enhancement was observed in *Scendesmus obligus* when grown in nitrate deficient media for 7 days [10].

IV. CONCLUSION

The present results suggest nitrogen starvation triggers lipid accumulation in *Chlorella pyrenoidosa*. The most effective method for enhancement in lipid production is grow in medium with KNO_3 0.05g/L which gives 12% increment in lipid content than control.

V. ACKNOWLEDGEMENT

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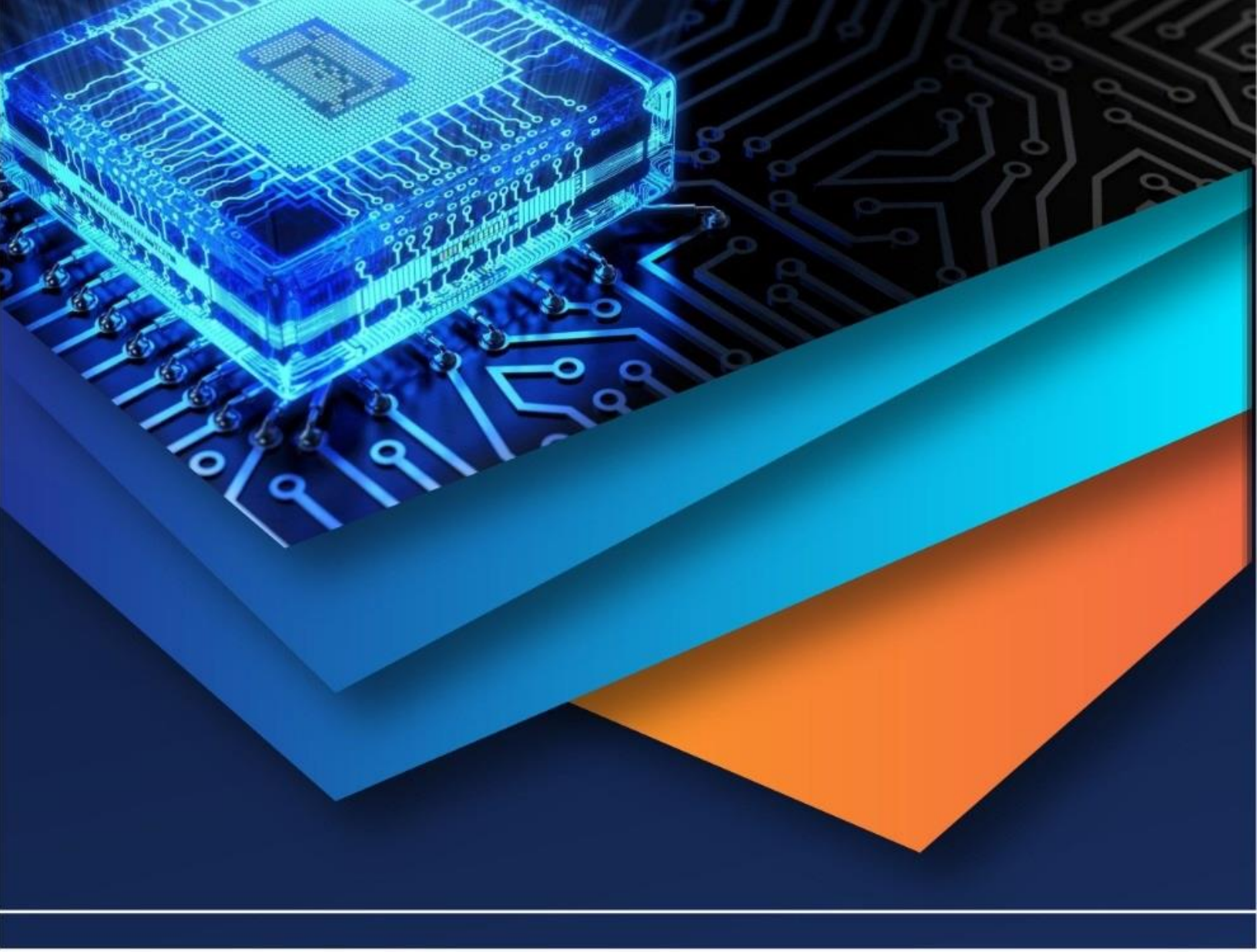

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