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# STUDIES ON THE ENHANCEMENT OF LIPID PRODUCTION IN *Chlorella pyrenoidosa*

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## ABSTRACT

Microalgae are emerging as the sustainable key source of green fuels and neutraceuticals. In the current study, a strain of *Chlorella pyrenoidosa* was isolated, identified and biochemically analyzed. Studies were undertaken to enhance its biomass and lipid productivities for sustainable biodiesel production. Growth was monitored in BG-11, BBM, Bristol and Fogg's medium as cell count, O.D, PCV and dry cell weight measurements. BG-11was found to be the most suitable medium. Specific growth rate was determined to be 10.64 mg/l/d in 25 days with a lipid productivity of 29.26 mg/l/d. Nitrogen stress was introduced by varying concentration of major nitrogen source in the BG-11 medium. Varied inorganic nitrogen source like urea, ammonium nitrate and ammonium sulphate had a significant effect on biomass and lipid productivities of algae. Ammonium nitrate enhanced the lipid production leading to a three fold increase in its yield. Growth rate was accelerated by 15.3 % with the supply of 5 % carbon dioxide.

**KEYWORDS**: ***Chlorella* sp., Specific growth rate, Inorganic nitrogen and Lipid productivity.**

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# INTRODUCTION

Algae are large, diverse group of simple, autotrophic organisms that exist as unicellular and multicellular forms.[1,2] Their presence in nature and significant role in fossil fuels formation is evident from evolutionary studies.[3] It is into this era, that mankind is exploring algal energy stored in the form of triglycerides, to convert it into biodiesel.[4,1] Food versus fuel conflicts with respect to agricultural crops,[5] increasing demand of fossil fuels,[6] and an alarming issue of global warming are few of the major challenges that can be addressed by algae. Microalgal feedstock serves as an eco friendly, carbon neutral, clean and green fuel.[7] Different algal species are being studied for fatty acid composition, [8,9] nutritional supplements, anti-oxidants, natural dyes and animal feed. [10] Chlorella, The earth's oldest living organism, is a naturally existing, pure whole food. It is known to reproduce asexually by dividing four times every 20-24 hours. Strains like *C. protothecoides, C. vulgaris, C. pyrenoidosa, C. sorokiniana, C. zofingiensis* and *C. minuitissima* [11,12,13] are reported to be suitable for large scale biomass production. Several Chlorella sp. are directly being used as a feedstock for anaerobic hydrogen and methane production.[14] It has also been reported to be used for methane production from defatted algal biomass.[4] Chlorella utilizes light energy at a higher rate (10-20%) for photosynthesis as compared to plants. Its nutritional value holds great importance in food industries. It is primarily used as a source for production of proteins and antioxidants. Open ponds are one of the effective methods for its large-scale production. Its cultivation in bubble column photobioreactors and air-lift photobioreactors has been studied extensively and is in the process for commercial implementation.[15] Biodiesel production from unicellular algal oils has received a wide attention due to its rapid growth rate, higher lipid productivity and ease of culturing in auto, hetero or mixotrophic cultivation[16]. Their biochemical characteristics, biomass yields, lipid productivities and fatty acid composition are largely governed by ecological and physiological environmental conditions. Under normal growth conditions, the lipid accumulation is lower (<40%). High oil content is always associated with very low biomass yields. The lipid production can be stimulated under stress conditions, like nitrogen, phosphorus, temperature, salinity, light gradients etc. The current study aims at characterizing a indigenous strain of *Chlorella pyrenoidosa* for maximization of its biomass and lipid production. The strain was isolated and cultivated in laboratory to study its growth rates in different media. The strain was exposed to different concentrations of sodium nitrate in BG-11 medium to study the correlation ration between biomass and lipid yields of this strain. Also, the effect of various other inorganic nitrogen sources and carbon dioxide ($CO_2$) was studied.

# MATERIALS AND METHODS

### *1. Isolation and Identification*

A strain of *C. pyrenoidosa* was isolated from fresh water bodies of Pezari Lake from Alibagh, Maharashtra, India. Monocultures were obtained by serial sub culturing of the sample and were microscopically studied for their morphology and mode of reproduction. The strain was identified based on the established monograms of unicellular microalgae.[17] It was cultivated in laboratory in the suitable sterile medium in Erlenmeyer flasks, closed polyethylene sacks and exposed to natural sunlight at the north side window at ambient temperature, with pH 6-7.5.

### *2. Media Optimization and Determination of Growth*

### *Media Optimization*

The algal cells were cultivated in BG-11, Bold's Basal (BBM), Bristol and Fogg's medium[18] to select suitability for rapid their growth and proliferation of *C. pyrenoidosa*. An inoculum of algal cells with cell count of 5-6 x $10^4$cells/ml was used as a starting culture. The pure cultures were preserved via cryopreservation, using 40 % glycerol as a cryoprotectant along with saline/ BG-11 medium and were maintained at -80 $^0$C.

***Determination of Growth***

Growth was monitored as total cell count by neubauer hemocytometer, optical density measurement at 540 nm, packed cell volume and as dry cell weight for four weeks and calculated using following equations:

***(a) Growth rate based on Cell count***

$GR_{Cell\ count}$ = μ= (In Cell count $_t$ – In Cell count $_{to}$) / t-$t_o$,

Where μ is specific growth rate, t' is cell count on 't' day and '$t_o$' is initial cell count

***(b) Growth rate based on O.D***

$GR_{O.D}$ = (In $O.D_t$ – In $O.D_{to}$) / t-$t_o$,

where $O.D_t$ is optical density at time and $O.D_{to}$ is optical density at starting of the batch culture.

***(c) Growth rate based on dry cell weight***

$GR_{Dry\ cell\ wt}$ = (In Dry cell $wt_t$ – In Dry cell $wt_{to}$) / t-$t_o$

where Dry cell $wt_t$ is the biomass at time 't' and Dry cell $wt_{to}$ is the biomass at starting of the batch culture.

***(d) Growth rate based on PCV***

$GR_{PCV}$ = (In $PCV_t$ – In $PCV_{to}$) / t-$t_o$

where $PCV_t$ is the packed cell volume expressed as percent at time 't' and $PCV_{to}$ is packed cell volume expressed at the start point '$t_o$' of the batch.

Doubling Time was calculated as, Doubling Time = ln2 / μ, where μ is the specific growth rate.

***3. Biochemical Analysis***

At the end of stationary phase, cells were separated by sedimentation / centrifugation at 5000rpm, 4 $^0$C for 5 min. The obtained biomass pellet was dried at 60 $^0$C in a hot air oven till a constant weight was obtained. The dried algal biomass was further grinded into a powdery form (about 3-6 microns size) for its biochemical characterization and maximizing its exposure to solvent systems, used for oil extraction. Evaluation of Biochemical Profile involved estimation of lipids[19] (Bligh and Dyer method with methanol: chloroform in 1:1 ratio), carbohydrates by Phenol Sulphuric method,[20] soluble proteins by Folin- Lowry method,[21] free hexoses sugar by DNSA method[22] and ash contents in a muffle furnace.[23] Lipid yield and lipid productivity were calculated as follows:

Lipid yield was expressed as percent of lipid contents in unit algal biomass.

Lipid Yields = (Weight of lipids obtained / Dry cell weight of algae).100

Lipid Productivity = (Biomass Yields. Lipid Yields) / 100

***4. Study of Nitrogen Stress***

***Sodium Nitrate Concentrations***

Stress was introduced by limiting and depriving the nitrogen source. The strain was studied for growth and lipid content in different concentrations of sodium nitrate ($NaNO_3$). BG-11 medium contains 1.5 g/l $NaNO_3$. The experiment was performed using 0, 0.3, 0.6, 0.9, 1.2, 1.5 g/l concentration of $NaNO_3$. Biomass and lipid contents were estimated after 25 days (stationary phase) of inoculation.

***Varied Inorganic Nitrogen Sources***

The effect of different inorganic nitrogen sources was studied by substituting sodium nitrate with urea, ammonium nitrate and ammonium sulphate at 1.5g/l concentration of each. Growth rate was observed every 24 hrs while the lipid contents were estimated after 24 days of inoculation, at stationary phase which accounted for maximum lipid accumulation.

***5. Study of Effect of Carbon dioxide ($CO_2$)***

Cultures were aerated continuously with a mixture of 1 %, 5 % and 10% concentration of $CO_2$ with air at the rate of 0.1vvm.

***6. Statistical Analysis***

All the experiments were performed in triplicates and the results were expressed as their mean with standard deviation. Probability, $P < 0.05$ has been considered with respect to α level of significance.

## RESULTS AND DISCUSSION

***1. Media Optimization***

*C. pyrenoidosa* was cultivated in BG-11, Bold's Basal, Bristol and Fogg's medium and growth was evaluated as cell count in all the media (Fig 1). BG-11 medium showed remarkably high growth rates (14.2 x $10^4$ cells / ml / d) as compared to BBM (2.68 x $10^4$ cells / ml / d), Bristol (5.2 x $10^4$ cells / ml / d) and Fogg's ( 3.6 x $10^4$ cells / ml / d)

medium and was therefore used as an optimum growth medium for further studies. The growth was slower till 20 days in BG-11 medium but a exponential increase was exhibited from 20 to 25 days. After the stationary phase (24-26 days), the growth rate started declining, leading towards death phase. Death phase was characterized by **a** decrease in cell counts, loss of pigmentation and reduced lipid contents (7 %). Later the culture turned into cellular debris.

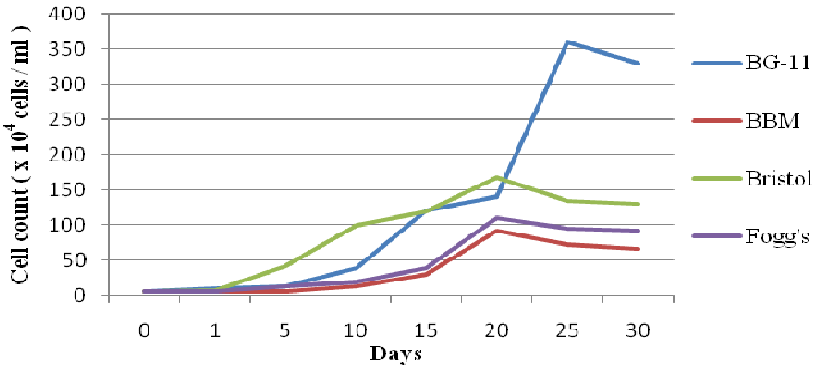


**Figure 1**
***Growth evaluation based on total cell counts in varied medium in C.* pyrenoidosa**

Growth was evaluated in all the media based on O.D. The same strain may exhibit varied OD values in different media as the size and density of cells changes with change in the media, thus finally affecting the O.D readings. Therefore, cell count was preferred for comparing the growth of algae in different media (Fig 2). The growth curves of *C. pyrenoidosa* determined by cell counts and O.D exhibited nearly similar growth patterns as depicted in Fig1 and 2. Growth rate of *C. pyrenoidosa* in BG-11 medium was also determined by packed cell volume and dry cell weight (Fig 3). Dry cell weight was considered for calculating the lipid yields and productivities.

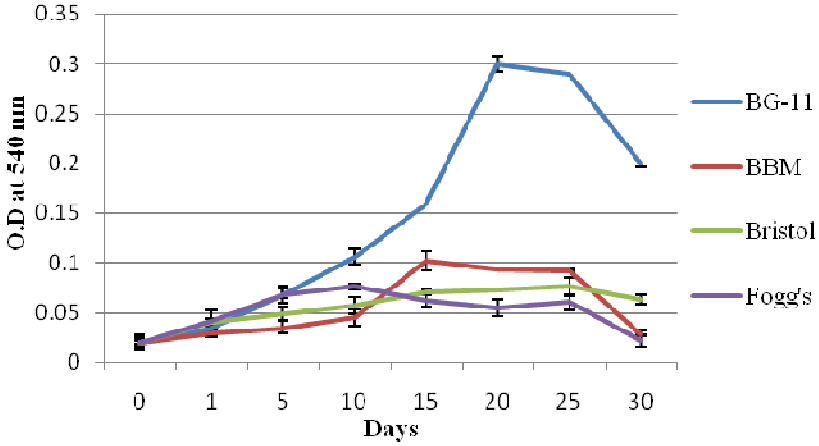


**Figure 2**
***Growth Curve of C.* pyrenoidosa *in Varied Medium determined by O.D***

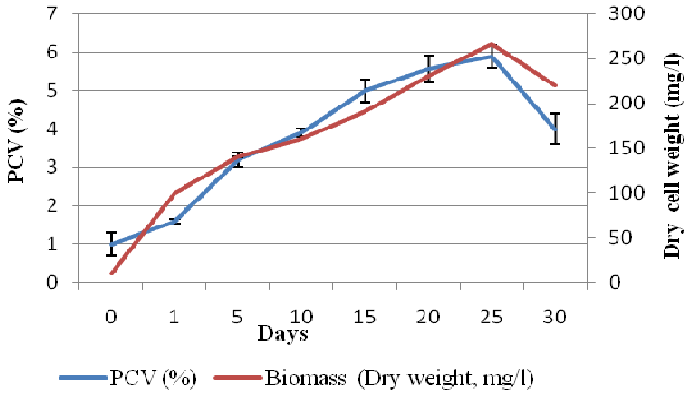


**Figure 3**
***Growth evaluation of C.* pyrenoidosa *in BG-11 medium determined by packed cell volume and dry cell weight***

### *2. Biochemical Analysis*

The studied strain of *C. pyrenoidosa* cultivated in BG-11 medium, had abundant reservoirs of protein (50 %). It exhibited 87 % of moisture contents. Carbohydrates constituted only 26.01 % of its biochemical profile (Table 1). Lipids contents were found to be around 11 %. To increase the lipid yields, the strain was further taken up for nitrogen stress studies. Mineral contents in the dried biomass were calculated to be 4% as ash contents and reducing sugars were detected in traces. The dried algal biomass, that is, dry cell weight was considered for calculating the lipid yields and productivities (Table 2). Biomass yield was calculated to be 0.266 mg/l in BG-11 medium (cells being harvested at stationary phase on 25th day). Based on biomass contents, biomass productivity/ growth rate was calculated to be 10.64 mg/l/day, with a doubling time of 1.62 hrs. Lipid yields were recorded to be 11 % (on dry cell weight basis) and lipid productivity was evaluated to be 29.26 mg/l/d.

**Table 1**
***Biochemical composition of C.* pyrenoidosa**

| Solids (%) | Moisture (%) | Protein (%) | Carbohydrate (%) | Lipid (%) | Mineral (%) | Reducing Sugar (%) |
|---|---|---|---|---|---|---|
| 12.71 ± 1.18 | 87.28 ± 1.18 | 50.02 ± 0.24 | 26 .01 ± 0.13 | 11.02 ± 0.94 | 4.04 ± 0.92 | 1.64 ± 0.53 |

***Results expressed as mean ± standard deviation***

**Table 2**
***Biomass and lipid productivities of C.* pyrenoidosa**

| Parameters | Obtained Values |
|---|---|
| **Biomass Yield** | 0.266mg/l |
| **Volumetric Biomass Productivity** | 10.64mg/l/day |
| **Doubling Time** | 1.62 hrs |
| **Lipid Yield** | 11 % |
| **Volumetric Lipid Productivity** | 29.26mg/l/d |

### *3. Study of Nitrogen Stress*

#### *Effect of Nitrogen Stress on Growth and Lipid Yields*

The strain was cultivated in BG-11 medium, which consisted of $NaNO_3$ (1.5g/l) as a major nitrogen source. Stress was introduced by lowering the concentration of $NaNO_3$ from 1.5 to 1.2, 0.9, 0.6, 0.3, 0 g/l. At each concentration, the biomass and lipid contents were recorded. A linear decrease in biomass and increase in lipid contents was observed with an increased degree of stress. At the

lowest concentration of 0.3g/l $NaNO_3$, lipid yields increased from 11% to 20 % but at the cost of decreased growth rates (Fig 4). But, the lipid productivity was highest (48.4 mg/l/d) at the lowest concentration of $NaNO_3$, therefore this concentration is the most significant for mass production of lipids from the current strain. It can be stated that the strain accumulated more lipids under stressed conditions. Growth of algae increased after a short lag phase of 2 days followed by a logarithmic phase and attained stationary phase in 25 days. In the absence of a nitrogen source (0 g/l $NaNO_3$), poor growth was observed. The result signifies the presence of nitrogen source as an essential requirement for the metabolism of algae and also for lipid production. The lipid contents increased at the rate of nearly 100 % at the lowest concentration (0.3 g/l) of $NaNO_3$ while the biomass reduced at the rate of 50 %. It has been reported earlier that with the increase in nitrogen stress condition in algae, the cell switches its metabolic pathway towards synthesizing more lipids[24]. Under normal condition, the cell synthesizes carbohydrate and doubles its number and increases in biomass contents. Therefore, if biomass increases, the lipid yields are decreased and vice versa. For sustainable production of biodiesel, an algal cell needs to have a balance, where its biomass contents and lipid contents are optimized for its economic feasibility. Volumetric lipid productivity plays a key role in defining the adaptability of optimized conditions for large scale oil production at economically feasible rates.

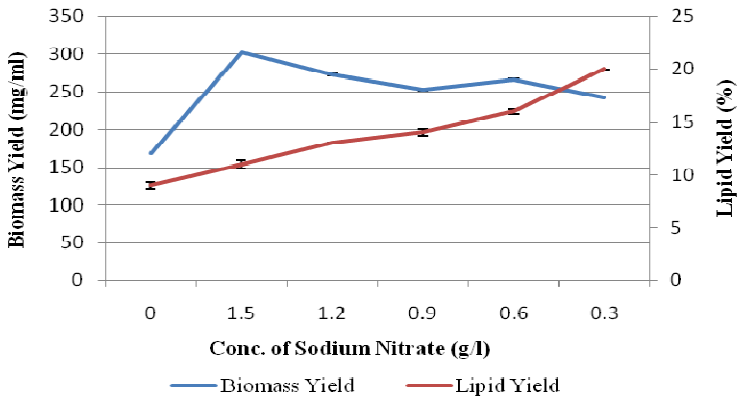


**Figure 4**
***Growth of Chlorella sp. in absence of nitrogen and in the presence of varied inorganic nitrogen salts in BG-11 medium***

***Effect of Different Nitrogen Sources on Growth and Lipid Yields***

There was a significant affect of various nitrogen sources on growth and lipid production in BG-11 medium. Ammonium nitrate led to a threefold increase in lipid contents. Urea accounted for about a two fold increase and ammonium sulphate led to two and a half fold increase in lipid contents. In the absence of nitrogen source, lipid and biomass yields were at the lowest, stating that nitrogen being an essential requirement for cellular growth and metabolism (Fig 5). Ammonium nitrate might not have been utilized by the strain, thus leading to nitrogen stress and a high lipid yield. For commercialization of algal biodiesel production from a strain, it also becomes necessary to evaluate its lipid productivity. Ammonium nitrate led to a threefold increase in lipid yields, 12 % decreased growth rate but at the cost of low lipid productivities (13.3 mg/l/d). In spite of its high oil yields, it is economically not feasible for mass production as depicted in Fig 5. On the other hand, though sodium nitrate had lower lipid contents as compared to ammonium nitrate, it was the most preferable nitrogen source in

BG-11 medium because of its higher lipid productive rates (29.26 mg/l/d). The volumetric lipid productivity is a significant factor in determining the suitability of media.

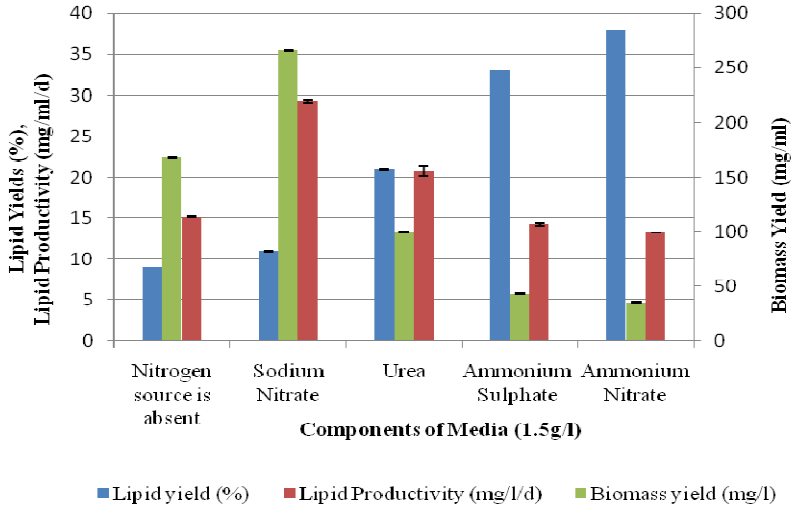


**Figure 5**
***Effect of nitrogen stress on biomass and lipid yields of Chlorella sp cultivated in BG-11 medium. 1.5 g/l is the normalized $NaNO_3$ concentration***

***4. Carbon dioxide Supplementation***

Supplementation of $CO_2$ from 1% to 5 % had a positive effect where as 10 % had a negative effect on the growth of microalgae (Fig 6). A 5 % supply of $CO_2$ led to 15.27 % raise in the growth rate of *C. pyrenoidosa* with a minor increase in its lipid contents (1.56 %). With $CO_2$ supplementation at 1%, it was observed to enhance biomass by 3.88 % and lipid yields by 0.6 %. It therefore states that 1 % and 5 % $CO_2$ supplementation leads to acceleration in the photosynthetic rate and thus enhances growth rates and biomass productivity at lower rates[25]. By increasing $CO_2$ to 10 %, there was a decline in the biomass by 87.5 % and lipid contents (7.5 %) stating the inhibitory effect of over dosage $CO_2$of in the studied strain.

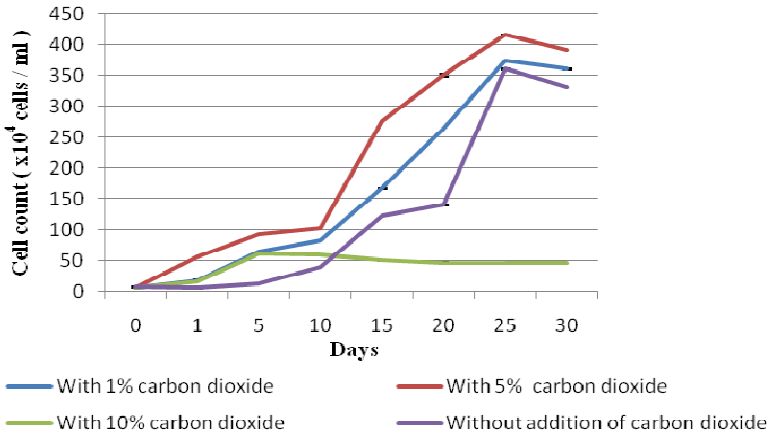


**Figure 6**
***Growth of C.* pyrenoidosa *at variable concentration of $CO_2$***

## CONCLUSION

*C. pyrenoidosa*, fresh water, unicellular algae was studied for its biomass and lipid productivities for sustainable biodiesel production. It showed highest specific growth rates in BG-11 medium. The strain was found to have lower lipids contents. Therefore, nitrogen stress studies were undertaken to enhance lipid yields and its productivity. Nitrogen starvation was introduced by varying the concentrations of sodium nitrate in the medium. At the second stage, sodium nitrate was replaced by varied inorganic nitrogen sources to study their impact on the productivity of the strain. Optimizing the concentration of $NaNO_3$ at 0.3g/l, resulted in enhancing lipid productivity to twofold with higher desirable volumetric lipid productivity. This ensures the effectiveness of this concentration for oil production at economically feasible rates. Addition of ammonium nitrate to BG-11 medium as a major nitrogen source led to three fold increase in lipid yields as compared to the $NaNO_3$. But, both the biomass and volumetric lipid productivities were lowered, which is not suitable for large scale productions in batch cultures. Therefore, $NaNO_3$ was found to be the best inorganic nitrogen source at 0.3g/l concentration in the BG-11 medium.

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