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Nitrogen replete stress condition for enhanced lipid accumulation in microalgae *Chlorella* sp.

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Abstract

Microalgae are regarded as an alternative feedstock for biofuel production from its lipids and its biomass, However, there are still several obstacles that must be overcome for the commercial production, such as reducing production costs. Therefore, in this study the microalgae *Chlorella* sp. was isolated, purified and applied nitrogen stress as replete condition. The *Chlorella* sp. observed specific growth rate of 0.032/hr and doubling time of 8 hrs with 50% lipid content. The different nitrogen ($NaNO_3$ and NH_4Cl) replete stress was applied and 4.4mM $NaNO_3$ concentrations increased the lipid accumulation content up to 53%, when applied in stationary phase for 62 hrs. However, the nitrogen stress on initial day's affected microalgae growth drastically, hence this study suggests that the nitrogen stress should be given at stationary phase. The protein profile of nitrogen stress condition revealed a specific protein. This result can be used in the commercial application for lipid production from microalgae eventually reduces the cost of microalgae biofuels.

Keywords: Biofuel, Lipid, Microalgae, Nitrogen Stress, Replete

1. Introduction

Renewable and sustainable energy is a big issue in the world and will continue to be so in the near future also due to the exhaustion of fossil fuel. Therefore, to overcome the crisis, the conversion of biomass oil to biofuel is presently increasing [1, 2]. Owing to the concerns being raised about the use of arable land and food crops for bioenergy production, research has been focused on alternative biomass sources. Among various biomass energy resources, biofuel extracted from microalgae have attracted attention all over the world [3, 4]. Microalgae are aquatic, eukaryotic and prokaryotic photosynthetic organisms, and are highly capable of efficiently utilizing solar energy and CO_2 to create biomass. Like terrestrial plants, microalgae produce neutral storage lipids in the form of triglycerides, long-chain fatty acids which are used in transesterification to produce biodiesel. They are capable of utilizing the nutrients in wastewater [5, 6] and, most importantly, they have high growth rates, nil requirement of arable land and contribute to CO_2 mitigation [3, 7]. For all these reasons, microalgae are attractive for meeting today's energy demands. However, microalgae produce more oil than other crops, but still biofuel from microalgae is not yet cost efficient [4]. There is an urgent need for high oil producing microalgae for sustainable biodiesel production. Many researchers engage in this field with diverse approach to improve the efficiency of biofuel production from microalgae at various sources, due to possible cultivation on a large scale. Currently biofuel production from microalgae is still far more expensive than fossil fuel because of low oil content, low yield of biomass, poor culture system, scale of production, and high cost of recovering oil. Assuming that the oil content of the algae is approximately 30 percent, the production cost would be ~$2.8 per liter of algal oil [3] equivalent to 156 rupees/liter. Importantly, this estimation does not include costs of converting algal oil to biodiesel, distribution and marketing costs, and taxes. Whereas, the petroleum and diesel price in India is 65 and 56 rupees per liter, as on date 30/11/2016, respectively. This creates a question in everyone's mind whether algal oil can be an economic source for bio-energy production?. For enhancing lipid accumulation in algae, several approaches have been used such as nitrogen deprivation [8], carbon dioxide inclusion [9] high salt content [10] pH, temperature, light intensity[9] [11] random mutagenesis [12] and enzymes [13]. One of the most typical methods for inducing cellular lipid accumulation during cultivation is nitrogen limitation. When microalgae are placed under nitrogen limitation, cells stop growing and cellular lipids start accumulating [14]. However, high cellular lipid content does not always guarantee the enhancement of lipid productivity, since the nitrogen limited condition reduce the cell growth rate or even a loss of biomass concentration [15]. Moreover, nitrogen limitation is not a universal stress factor that stimulates lipid accumulation, although lipid synthesis is induced in many microalgae but not all. The dependence of lipid synthesis on nitrogen limitation is species specific and sometimes

even strain specific [16]. For some microalgae, lipid synthesis does not respond to nitrogen limitation, and in some cases, larger lipid production is observed at higher nitrogen levels [17, 18]. The present study investigated the influence of nitrate replete condition on lipid accumulation in the microalgae *Chlorella* sp. In order to enhance the lipid productivity, culture was supplied with different nitrogen sources at replete condition in different growth stage. Then the biomass concentration, lipid contents and total cellular protein was analyzed and compared.

2. Materials and Methods

2.1. Isolation of Microalgae, purification and identification

The microalgal water samples were collected in a sterile container from Pallavaram lake, Chennai and inoculated into 500ml beaker containing 250 ml of TAP medium. The sample was incubated for 10 days at 25^{o} C under continuous light. After 10 days the algal samples were checked for the bacterial contamination in nutrient agar medium by streaking the algal sample. In order to purify the microalga, the isolated microalgae was incubated with AAS (Antibiotic Antimycotic Solution-contains 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml) solution at 2x concentration for different time to kill the bacterial and fungal contamination. The purified microalga was identified using compound microscope and confocal laser scanning microscope.

2.2. Determination of specific growth rate

The 1 mL freshly grown microalga (5 days old) was inoculated into the 100 ml TAP medium and incubated for 10 days at 25^{o} C under continuous light. 2 mL samples were collected for every 24 hrs and observed OD_{680} nm and cells were diluted and counted using hemocytometer.

The specific growth rate was calculated by the following equation

$$\mu = (\log N2 - \mathrm{Log}N1)/(T2 - T1)$$

µ= specific growth rate constant
N1=cell concentration at the end of the experiment period
N2= cell concentration at the beginning of the experiment period (zero day)
T=hrs
Doubling time was calculated using following equation

$DT = T_2\ (OD) - T_1\ (OD)$

2.3. Nitrogen replete treatment

To check the effect of Nitrogen replete stress on microalgae for its lipid accumulation, the isolated microalgae was grown in TAP media with different N source as NH_4Cl, $NaNO_3$. The concentration was given as 1x, 5x and 10x. The 1x is actual concentration of nitrogen in TAP media which is 7 mM (37.5mg/100 ml) for NH_4Cl, and 0.9 µM (7.5 mg/100 ml) for $NaNO_3$. The another treatment as adding Nitrogen stress on the 5th day of the growth and incubated for 2 days in 25º C under continuous light. The growth was analyzed as OD observation and lipid was measured by gravimetric method.

2.4. Lipid extraction and quantification

Microalgae was centrifuged, dried and weighed about 50 mg, then added 3 mL of chloroform:methanol (2:1 v/v) and vortexed for 20 min. Then, 2 mL of chloroform was added and vortexed for 10 min. Furthermore, 1 mL of distilled water was added, mixed and centrifuged at 4000 rpm for 10 min. The bottom layer was collected and transferred into a pre-weighed tube. The solvent was evaporated by dried at 80°C for 30 min, cooled and weighed, and the lipid content was calculated using the following equation

$$\text{Total lipid (\% dry weight)} = \frac{(W2 - W1)\ x\ V1}{Wb\ x\ V2}\ x\ 1000$$

where, W1 is the weight of empty tube (g), W2 is the weight of tube with dried lipid (g), Wb is the weight of biomass (g), V1 is the total volume of chloroform added (ml), and V2 is the volume of chloroform transferred to the empty tube (ml).

2.5. Protein characterization

Total Protein content of the samples was estimated qualitatively using SDS PAGE analysis followed the methods of Laemmli [19] For extraction of proteins, the samples were homogenized with lysis buffer containing 0.5M Tris-HCl, 8M Urea, 5% (w/v) SDS, 20% (v/v) Glycerol and 10% (v/v) β-Mercaptoethanol; final pH 6.8 and centrifuged at 4°C for 20 min at 10,000 rpm. Protein extract was used for SDS-PAGE analysis, using a 12% polyacrylamide gel containing 0.1% SDS. Gels were run at 20°C at a constant current of 15 mA, for approximately 4 hrs. Gels were stained with 1% Coomassie brilliant blue, for protein visualization.

3. Results

The Fig. 1 shows the growth of microalgae and its bacterial contamination. The Pallavaram water samples were shown copious growth of microalgae in TAP media (Fig. 1A) after 5 days of incubation. The microalga was shown bacterial contamination in nutrient agar (Fig. 1B) whereas no fungal contamination. As in this study needs pure culture for further experiment therefore the microalgae was purified by adding 2x AAS solution for different contact time. It was shown 2h contact time killed all bacteria as no bacterial growth was noticed 2h and afterwards. However, more incubation with AAS solution shown stunned growth as observed reduced OD therefore we selected 2h for purification of microalgae from bacterial contamination. The pure culture of microalgae was streaked in TAP agar medium and isolated a single colony for further studies

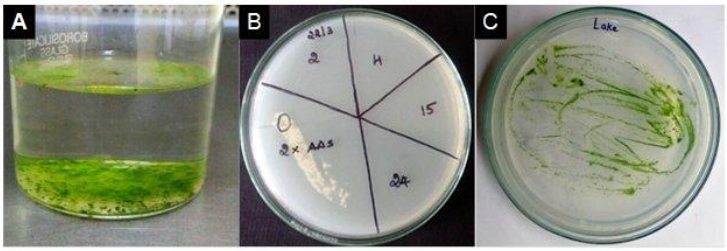

Fig. 1. The microalgal growth in TAP medium (A), the nutrient agar showing the bacterial contamination in microalgal samples and its purification with 2x AAS solution (B), purified microalgal growth in the TAP agar medium

The pure culture of microalgae was checked for its identification under compound and confocal microscope (Fig. 2). It was revealed that the *Chlorella* sp. as it was shown spherical in shape with 5µm in diameter and without any flagella and horse shoe shaped chlorophyll (Fig. 2B).

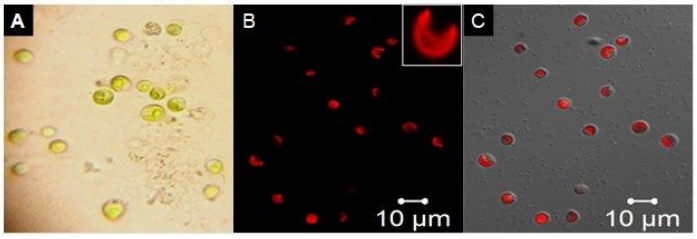

Fig. 2. Micrograph of pure cultures of microalgae isolated from Pallavaram lake water obtained with photomicroscope (450x) (A), confocal laser microscope (B and C). The inside Figure showing the cup shaped chlorophyll.

The isolated *Chlorella* sp. was checked for growth characteristics using colorimetric method at 680 nm and cell count for up to 10 days (Fig. 3). The OD value gradually increased from initial at 0.102 up to 2.789 on 10^{th} day. The maximum cell number was observed on fifth day of incubation at 3.14×10^{7} and also from there stationary phase begins under normal condition without any nutrient stress. The specific growth rate was found to be 0.032 per hour and the doubling time was calculated through the OD experiment as 8 hours in logarithmic stage.

Specific growth rate = $\frac{7.19-6.42}{72-48} = 0.032/\text{hr}$

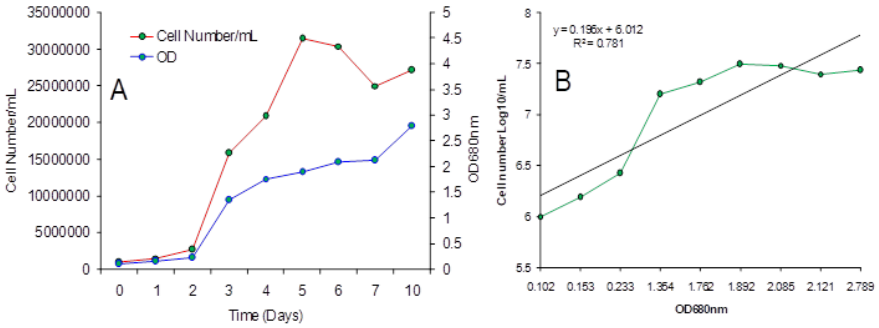

Fig. 3. Growth curve (A) and correlation graph of OD vs cell number (B) of the microalgae *Chlorella* sp. Isolated from the Pallavaram lake sample

The nitrogen stress as replete condition in TAP media clearly shown adverse effect as there was no growth in 5x and 10x concentration of NH_4Cl while there was reduced growth in $NaNO_3$ and it was toxic to the algae when growing in initial days of the incubation. Whereas, the 1x concentration of NH_4Cl did not affected the growth as expected because that is the optimum concentration (37.5 mg/100 ml) in TAP media. Therefore the nitrogen stress condition in the initial stage of growth is not advisable.

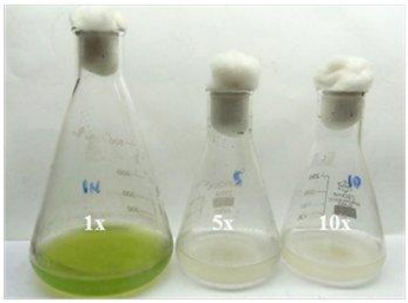

Fig. 4. *Chlorella* sp. growth after 5 days of incubation with stress (NH4Cl in three different concentrations 1x (7 mM), 5x, 10x) in repletion condition.

Therefore, two different sources of N replete stress (5x concentrations of NH_4Cl and $NaNO_3$) were applied to the culture on the 5th day when the stationary phase begins. OD and lipid content were analyzed after 62 hours of N stress in replete condition. Results showed a considerable increase in algal growth compared to the normal growth as OD was increased at 3.90 and 3.99 in NH_4Cl and $NaNO_3$, whereas the in TAP observed at 2.12, respectively. Microalgae are known to accumulate cellular lipids under nitrogen limited environment [12, 15]. But in this study the microalgae were treated N replete condition and observed 5x $NaNO_3$ stress condition shown highest lipid content of 53.2 % when comparing with 5x NH_4Cl (50.5%) and 1x NH_4Cl (50.1%) (Fig. 5). The result (Table.3) clearly shows that the N-stress has improved the lipid accumulation upto 3.2% when compared to control 1x sample, whereas 5x NH_4Cl shows only 0.4% increase when comparing with control. Therefore, this study suggests that the 5x $NaNO_3$ is a suitable N stress for lipid accumulation. The maximum yield of lipid have been gained through supplying sufficient nitrogen conditions, but not through deficient conditions [7]. The nitrogen stress condition in the initial stage of growth is not advisable, whereas addition of N stress replete condition on 5^{th} day at stationary phase shows maximum production of 53.2% lipid content with 5x $NaNO_3$ concentration

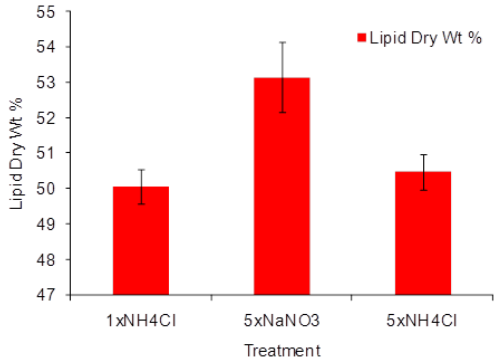

Fig. 5. Lipid content of *Chlorella* sp. under nitrogen stress (replete) condition.

Cellular process and lipid accumulation is controlled by proteins present in the organisms. Therefore, SDS-PAGE analyses were conducted to investigate the presence of intrinsic protein in the microalgal strain *Chlorella sp.* under nitrogen replete condition. The Fig. 6 had shown the total protein profiles of SDS-PAGE gel from the *Chlorella* sp. which was given the N replete stress on the 5th day of incubation with 5x concentration of NH_4Cl and $NaNO_3$. It was clear that 5x $NaNO_3$ treatments has more specific protein than the control 1x and 5x NH_4Cl samples and this protein is responsible for lipid accumulation in algal cells. In future this protein can be analyzed further and the lipid accumulation mechanism can be revealed by metabolic engineering method.

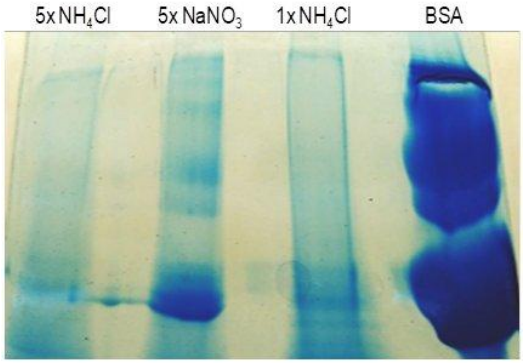

Fig. 6. Total protein profiles of *Chlorella* sp. grown in nitrogen replete condition

4. Discussion

The freshwater microalgae *Chlorella sp*. was isolated, purified and characterized in terms of its growth and lipid content. Generally, the water samples for microlage isolation will have bacterial and fungal contamination, and it's not easy and tedious to purify and get axenic culture [20, 21]. Here we adapted a method of AAS solution treatment at 2x concentration for 2h has given pure algae and killed bacterial and fungal contamination. The isolated *Chlorella* sp., observed specific growth rate, generation time and lipid content were found to be 0.032 per hour, 8 hrs and 50% respectively in normal culture sample. Many microalgae species were reported to decrease their cell growth rate significantly under nitrogen deprivation. However, some microalgae species were reported to sustain their growth by utilizing intracellular nitrogen storage, such as chlorophyll, by conversion to proteins, nucleic acid, and cell wall materials [22]. Is it possible to increase the lipid accumulation by giving N replete condition?, to know the fact in this study used different N source as NH_4Cl and $NaNO_3$ was added at 5x concentration. Interestingly, It was revealed that the biosynthesis and accumulation of lipids of *Chlorella* sp. was maximally recorded at 53.2% when N stress ($NaNO_3$) was introduced at stationary phase. After 62 hrs of incubation, the N stress gives 3% more lipid as when it compared with the corresponding control sample, thus the results indicate that nitrogen replete condition is a feasible tool for the production of lipid contents. Mostly, microalgae are known to accumulate cellular lipids under nitrogen limited conditions, by converting the flow of fixed carbon from protein synthesis to lipid synthesis [14, 23]. However, such

limited nutrient stressful conditions reduce microalgal growth, resulting in a decrease of lipid productivity. For this reason, nitrogen sufficient conditions have been regarded as a more economical approach for improving lipid productivity, rather than nitrogen deplete conditions [24]. Interestingly, the present study, higher lipid content was obtained under nitrate replete conditions, indicating that enhanced lipid productivity could be achieved. In accordance to our study, previous literature was shown highest lipid content and lipid productivity under N-replete conditions than N-deplete conditions in *Chlorella pyrenoidosa* [25]. Xu et al., (2001) and Feng et al (2011) found that the lipid content of a marine microalga *Ellipsoidion* sp. increased with increasing nitrate concentrations, and the maximum lipid content was obtained at the highest nitrate concentration [17, 18]. According to the review study by Griffiths and Harrison (2009), among 24 different microalgal species including green microalgae and cyanobacteria, seventeen species showed lipid accumulation under N deplete conditions, while seven species showed an increase of lipid content under N replete conditions [16]. Therefore it is thought that the responses of algal cells to nitrogen deplete/replete conditions are species specific as well as an intrinsic characteristic. Moreover, N replete condition in initial stage of growth was given detrimental effect on the culture as no growth observed, therefore it is advised to add N replete condition on start of stationary phase. The same result was reported in *Tetraselmis* sp., When the cells were cultivated through a two-stage culture process in which lipid accumulation was induced by a nitrate surplus condition with nitrate concentration of 8.82 mM in the second stage, enhanced lipid productivity of 47.3 mg/L/day was achieved [26]. Some microalgae species were reported to show cell growth inhibition at the high N concentration of 15 mM [22]. Moreover, it was found that lipid content increased when nitrate was present in the medium, but the lipid content did not increase after the nitrate was depleted. Nitrogen replete condition could trigger the protein which is responsible for lipid accumulating. Lipid producing proteins are stored in the algal cells which have been characterized by SDS PAGE method and lipid accumulating protein was seen in 5x $NaNO_3$ treatments. The protein profile of nitrogen stress condition revealed that a specific protein is produced by N stressed microalgae which leads to lipid accumulation. This result can be used in commercial applications for lipid production from microalgae and gene manipulation for strain improvement. The accumulation of lipids under N-repletion was attributed to the enhanced activity of acetyl-CoA carboxylase or other key enzymes related to the conversion of poly saccharides into lipids [18, 27]. Therefore, in our study the specific protein band might be the acetyl-CoA carboxylase or other key enzymes and further study is going on to identify the specific protein band.

5. Conclusion

The freshwater microalgae *Chlorella* sp. was isolated, purified and characterized in terms of its growth and lipid content. 2x AAS treatment for 2 hrs can give pure cultures of microalgae from the bacterial and fungal contamination. It was found specific growth rate at 0.032 per hr and 8 hrs for generation time, and 50% lipid content. The nitrogen ($NaNO_3$) replete (4.4 mM) growth condition increased the lipid accumulation up to 53%, when applied in stationary phase for 62 hrs. However, the nitrogen stress on initial day's affected microalgae growth drastically, thus suggested stress condition should be induced on stationary phase. Other nitrogen sources of NH_4Cl did not show any significant lipid enhancement. The protein profile of nitrogen stress condition revealed a specific protein in stressed microalgae. Hence the specific protein study will lead to molecular mechanisms of lipid accumulation and strain improvement. This result can be used in the commercial application for lipid production and could improve other biopolymers from microalgae.

6. Acknowledgments

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Conflict of Interest: Conflict of interest declared none.

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