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Optimization of Concentration of Two Different Nitrogen Sources to Enhance Biofuel Potential of *Chlorella Pyrenoidosa*

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Abstract: **In current scenario 'Oilgae' have been used as a promising source for production of biofuel. *Chlorella* is one of widely used microalgae for energy production and human consumption. The present study investigated the effect on biomass and lipid accumulation in *Chlorella pyrenoidosa* by cultivating in Fogg's media using ammonium chloride and urea as nitrogen source. The result revealed that as concentration of N-source in media has been increased the biomass content increased but lipid content decreased and vice versa. Furthermore, at same concentration of ammonium chloride and urea, the lipid and biomass content was recorded higher in urea in comparison to ammonium chloride. The optimum concentration of urea which gave maximum biomass of 5.467 ±0.034mg/ml was 0.5g/L and maximum lipid content of 30.2 ±0.20% dry weight was recorded at 0.1g/L. At high concentration of ammonium chloride growth rate decreased due to ammonium toxicity in algal culture. The optimization of urea concentration could be proved a new cultivation strategy to enhance biofuel potential of *Chlorella pyrenoidosa.***

Keywords: **Biofuel, *Chlorella pyrenoidosa*, Microalgae, Urea, Biomass, Lipids.**

I. INTRODUCTION

Urbanization and industrialization has put a burden on energy resources as well as incontestable damage to environment due to emission of greenhouse gases. There is an urgent need of safe, green and sustainable sources of energy as biofuels. (Meher *et al*., 2006). Biofuel or techanically biodiesel is the monoalkyl esters of long -chain fatty acids (FAs) which is derived from renewable feed stocks i.e. biomass. (like animal fats, plant oils, or other lipids) (Meher *et al*., 2006; Moser and Vaughn, 2012). in recent years Biodiesel is receiving increasing attentions because it is non-pollutant, non- toxic and renewable form of energy which can help to get solution of energy crisis(Chisti, 2007). At present food and oil yielding crops like maize, sugarcane, soyabean and rape seeds are most easily accessible raw material for biodiesel production. (Amaro, *et al*., 2011). Using of food crops for fuel production can have negative impact on food consumption protection. So there should be alternative resources for production of biofuel. (Makareviciene, *et al*., 2011). Algal biomass is usually rich in starch and can be easily fermented to produce liquid biofuels as biobutanol and bioethanol. Algal oils or oilgae can be converted to diesel, and jet fuel using advanced existing technology (Chisti, 2007; Demirbas, 2011).So use of microalgae to obtain energy rich algal oil is an alternative way for commercial production of l biofuel rather than use of food crops. The microalgae have average lipid content between 1 and 70% (Spolaore, *et al*., 2006; Lin, *et al*., 2008 and Chisti, 2007). A range of microalgal cultivation conditions could be used in order to induce significant amount of lipid production in microalgae (Sheehan *et al*., 1998). Even the stress conditions such as high light intensity, change in wavelength of light, nitrogen deprivation, high salt concentration, and low temperature conditions causes increase in lipid content commonly ranging from 30% to 70% of the total dry cell weight. Among all these factors, nitrogen is considered to have a strong influence on the growth and metabolism of lipids in various microalgae (Hsieh and Wu, 2009; Liang, *et al*., 2009).

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II. MATERIALS & METHODS

A. Sample collection:

The sewage water Samples were collected from Waste Water Treatment Plant Kholriwal, Jalandhar. The algae were isolated from sewage water the by step dilution method. By streaking method each dilution was poured on agar solidified Fogg's media (Fogg 1949). Different types of microalgae were observed with the help of compound microscope. *Chlorella pyrenoidosa* was identified and selected for further experimental study.

B. Culture of algae:

Starter culture of *Chlorella pyrenoidosa* was prepared by culturing them in culture flasks with 250 mL of Fogg's medium under controlled temperature at 25^0C, providing 16:8 light/dark conditions. The cultures were grown in a photobioreactor. This was taken as control culture. The culture flasks were shaken manually with hand three to four times daily to prevent sticking of algal cells with the walls of flasks. All glass wares and the media was sterilized before starting the inoculation.

C. Culture of Chlorella pyrenoidosa in different N-sources (Urea and ammonium chloride):

50 ml isolate of *Chlorella sp.* was inoculated in 250 ml of Fogg's media. Different concentration of urea and ammonium chloride as nitrogen source was used. The concentrations used in present study were 2.0g/L, 1.5g/L, 1.0g/L, 0.5g/L, 0.1g/L, 0.05g/L and without nitrogen.

D. Dry Cell Weight:

In order to determine dry weight of culture, 3ml of culture sample was taken in centrifuge tube and was centrifugated at 2500 rpm for 10min. The supernatant was discarded and wet algal mass was dried in oven at temp 80^0C for 2hours to get constant weight. After getting the constant weight the weight of algal biomass at end was subtracted from weight before drying.

$DCW= W_2-W_1$

W_2= weight of wet algal biomass before drying

W_1= weight of dry algal biomass after drying.

To calculate biomass content the regression equation was used. The conversion factor was calculated by plotting Dry Cell Weight (DCW) versus OD at 660nm. The linear regression equation was obtained as:

$Y= b.X+ a$ (Equ 1).

Where Y is biomass content (g/L) of microalgae cells and X= O.D at 640nm and 'b' is slope of line and 'a' is intercept of line.

E. Lipid content measurement:

Lipid content in microalgae was measured using Bligh and Dyer method (1959). 3ml. algal suspension was centrifugated at 3800 rpm for 10 minutes. The wet weight estimation of algae pallet was done gravimetrically. Then algal paste was dried at 80^0C for 2hours to get the constant weight. To 1g of dried algal biomass 2mL of chloroform and 1mL methanol was added. This suspension was left unobstructed for 24 hours at 18^oC. After 24 hours the solution was vortxed for 1 min. Again 1 ml of chloroform and 2 ml of water was added and agitated for 2-3 min. For complete layer separation whole content was centrifugation at 2000 rpm for 10min. With the help of glass syringe the lower layer with lipids was extracted and transferred to pre weighed vial (W1). The solvent was entirely evaporated and vial was again weighed (W2). Lipid content was calculated as W1 -W2 and expressed as % dry cell weight.

Statical analysis:

All experiments were performed in triplicates and data of biomass and lipid content subjected to ANOVA test using SPSS. The results were expressed as mean$\pm$ standard deviation. N=3.

III. RESULTS AND DISCUSSION

Nitrogen is an indispensable nutritional facet for the growth of algae. Algae are able to utilize ammonia, nitrate and organic sources of nitrogen such as urea. (Tepe *et al.*, 2006; Piorreck *et al.*, 1984; Goksan *et al.*, 2011). Our previous study proved that in microalgae, with decrease in concentration of nitrate the lipid production increases (Kaur *et al.*, 2016;

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Kaur *et al.*, 2017). Many studies also reported that growth of microalgae is also effected with different nitrogen sources used. (Jeansfils *et al.*, 1993; Fidalgo *et al.*, 1998; Sheekh *et al.*, 2014). The growth profile of *Chlorella pyrenoidosa* autotrophically in Fogg's medium with different nitrogen sources (urea and ammonium chloride) was studied. The biomass of *C. pyrenoidosa* increased from day 1 starting from lag phase to day 20 stationary phase and decline phase till 24 days. Fig.1 and Fig.2 showed growth curve of *Chlorella pyrenoidosa* in different concentration of urea and ammonium chloride respectively. From Fig.1 it has been depicted that initial growth of microalga was almost similar even at different concentration of urea. Urea concentration directly affects cell division so growth of microalgae is strongly influenced by concentration of urea in culture media. In lag phase of growth; biomass production is quite same even at different concentration of urea. There was increase in biomass content with increase in urea concentration up to 0.5gL-1. Urea quickly breaks into ammonium & the additional CO_2 is used by microalgae in photosynthesis. So rate of growth is also enhanced (Goswami *et al.*, 2009; Battah *et al.*, 2010). From Fig. 1 it is obvious that higher urea concentration (>0.5g/L) led to decrease in biomass. This could be attributed to fact that there is ammonium toxicity in culture media with high concentration of urea. Excess of ammonium in culture media causes the ammonium toxicity which causes drop in pH of media also. This will disrupt the cell constituent resulted in chlorophyll decomposition (Choochote *et al.*, 2010). These both factors inhibit the synthesis of ATP in chloroplast which ultimately leads to inhibition of algal growth at higher concentration of urea (Xin *et al.*, 2010; Zhu *et al.*, 2007; Takagi *et al.*, 2000). While at lower concentration (i.e.<0.5g/L) urea increases growth due to easy utilization by algal cells and therefore enhances vegetative growth of the algae (Sayed *et al.*, 2008).

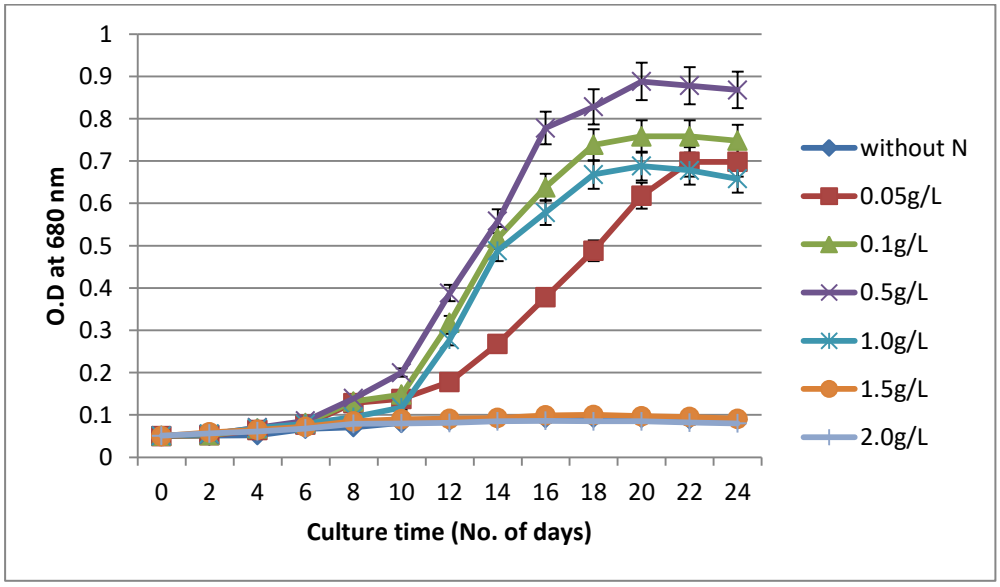

Fig 1: Growth curve of *Chlorella pyrenoidosa* in different concentration of urea.

In table 1 the biomass and lipid content at different concentration of urea has been presented. The optimum concentration of urea which gave maximum biomass of 5.467 ±0.034mg/ml was 0.5g/L. As concentration increases the biomass content decreased. At 2.0g/L of urea the biomass content obtained was 5.48±0.028 which is even lowest than any other concentration used.

Table 1: The biomass content, specific growth, biomass productivity and % lipid content in C.pyrenoidosa in various concentration of Urea. The values are mean± standard deviation. N=3

Urea concentration (g/L)	0	0.05	0.1	0.5	1.0	1.5	2.0
Biomass content mg/ml	0.591 ±0.024	4.299±0.020	4.667 ±0.03	5.467 ±0.034	4.238 ±0.04	0.634 ±0.018	0.548 ±0.028
Lipid Content (Mass %)	17.3±0.25	21.8 ±0.35	30.2 ±0.20	27.1 ±0.26	21.4 ±0.32	18.2 ±0.50	15.6 ±0.32

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Fig. 2 showed growth curve of *C. pyrenoidosa* in different concentration of ammonium chloride. Which depicted that initial growth rate of microalga was almost similar at different concentration of ammonium chloride. There was a sharp increase in biomass concentration as observed from O.D was noticed at concentration increases from nil to 0.5g/L around 8-10 day which is considered to be as exponential phase of algal growth. In subsequent period (6-17 days) as concentration of ammonium chloride increased in medium i.e. >0.5g/L the algal biomass also increased which could be depicted from O.D of culture media, because ammonium can be easily metabolized then nitrate and nitrite.

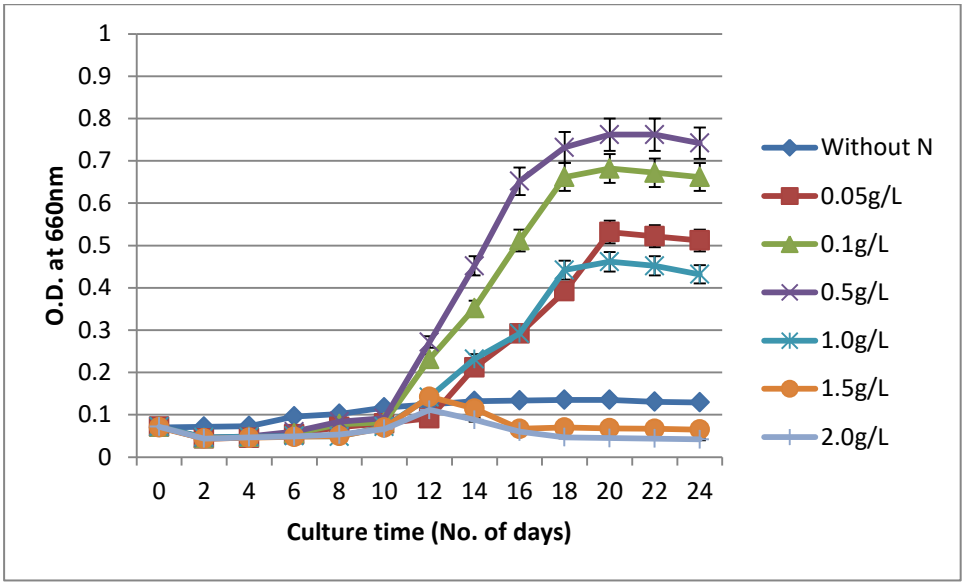

Fig 2: Growth curve of *Chlorella pyrenoidosa* in different concentration of ammonium chloride.

In table 2 the biomass and lipid content at different concentration of ammonium chloride has been presented. The optimum concentration of ammonium chloride which gave maximum biomass of 3.05 ±0.018 was 0.5g/L. as concentration increases the biomass content decreased. At 2.0g/L of ammonium chloride the biomass content obtained was 0.55±0.010mg/ml which is even lower than media without nitrogen source.

Table 2: The biomass content, and % lipid content in C.pyrenoidosa in various concentrations of Ammonium chloride (NH_4Cl). The values are mean± standard deviation. N=3

NH_4Cl concentration (g/L)	0	0.05	0.1	0.5	1.0	1.5	2.0
Biomass content g/L	0.594 ±0.022	2.12 ±0.017	2.52 ±0.030	3.05 ±0.018	1.86 ±0.02	0.64 ±0.030	0.55 ±0.019
Lipid Content (Mass %)	17.3±0.25	17.9±0.30	18.1±0.22	18.5±0.24	17.6±0.26	16.8 ±0.30	15.4 ±0.30

At very high concentration there is oversaturation of ammonia in media which leads to decrease in pH of media which does not allow diffusion of ammonia in microalgal cells. Most of ammonia remains unutilized in media and lowering of pH damages ATP synthesis machinery of microalgal cells. So growth is retarded and biomass content decreases. Our result agrees with earlier studies performed (Shameera *et al.*, 2012; Chang *et al.*, Li *et al.*, 2001).

Fig. 3 illustrates effect of different concentration of urea and ammonium chloride on lipid production in microalga *Chlorella pyrenoidosa*. It has been observed a general trend in both cases that with the increased concentration of nitrogen source in media lipid content decreased. In media with urea as nitrogen source, maximum lipid content obtained was 30.2 ±0.20% of dry cell weight at concentration of 0.1g/L. While in case of media with ammonium chloride as nitrogen source maximum lipid content recorded was 18.5±0.24% of dry cell weight at concentration of 0.5g/L.

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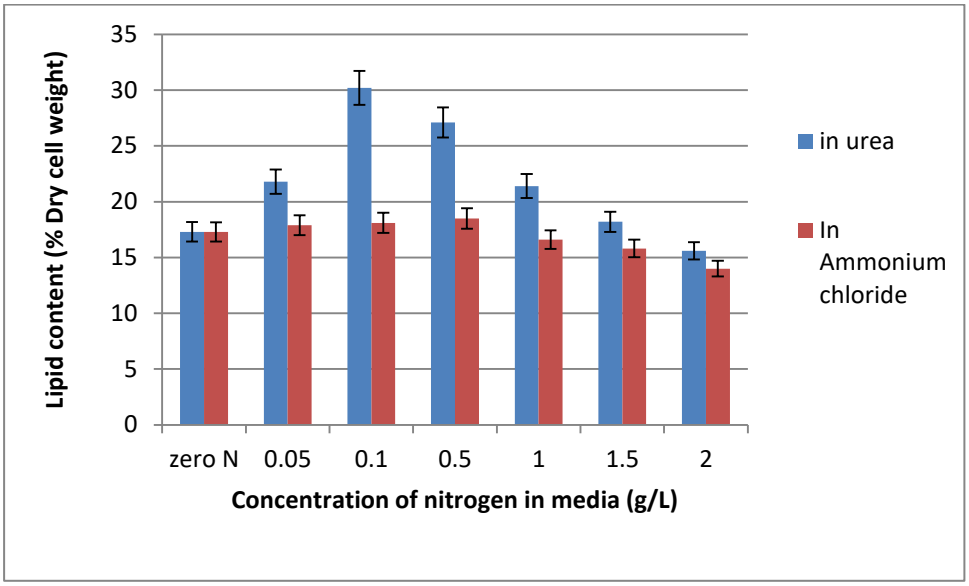

Fig 3: Effect of different sources of nitrogen at various concentrations on lipid production in *Chlorella pyrenoidosa.*

In nitrogen deficient media, algal cells are producing more fatty acids as compared to nitrogen rich media. This could be due to decrease in rate of photosynthesis in nitrogen starvation conditions which leads to lower glucose content in microalgal cell. So rate of acetyl Co-A synthesis also get lowered which directly retards synthesis of Mallyl Co-A and algal cells start accumulating carbon metabolites into more lipids (Gouveia and Oliveira *et al.*, 2009; Li *et al.*, 2001). If we compare lipid content in Chlorella sp. growing in media with ammonium chloride and urea as N-source, it has been perceived that urea enhance lipid accumulation as compared to using ammonium chloride. Urea splits into CO_2& ammonium and this additional CO_2 not only enhance growth of microalgae but also delivers excess carbon flux for lipid production (Chen *et al.*, 1991; Martinez and Orus 1991). In our present study the optimum concentration of urea was 0.1g/L with highest lipid content i.e. 30.2 ±0.20% of dry cell weight and biomass 4.667 ±0.03 mg/ml. Our result is in agreement with earlier studies performed (Torre *et al.*, 2004; Hsieh *et al.*, 2009; Danesi *et al.*, 2002). Moreover urea was reported best nitrogen source for culturing of *Chlorella* (Torre *et al.*, 2004; Hsieh *et al.*, 2009; Danesi *et al.*, 2002; Lin, *et al.*, 2008; Choochote *et al.* 2011; Kaur *et al.*, 2017).

IV. CONCLUSION

In conclusion, the results suggest that nitrogen starvation treatment and an optimized concentration of urea is considered to be superlative cultivation strategy to enhance biofuel potential of Chlorella pyrenoidosa. The most suitable and effective nitrogen source for *Chlorella pyrenoidosa* is urea which increases biomass content with high lipid content. Urea is cheap and easily available source as compared to other carbon and organic sources and is able to give desirable result at very low concentration.

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