World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:8, No:8, 2014

Statistical Optimization of Medium Components for Biomass Production of *Chlorella pyrenoidosa* under Autotrophic Conditions and Evaluation of Its Biochemical Composition under Stress Conditions

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***Abstract*—**The aim of the present work was to statistically design an autotrophic medium for maximum biomass production by *Chlorella pyrenoidosa* using response surface methodology. After evaluating one factor at a time approach, K_2HPO_4, KNO_3, $MgSO_4.7H_2O$ and $NaHCO_3$ were preferred over the other components of the fog's medium as most critical autotrophic medium components. The study showed that the maximum biomass yield was achieved while the concentrations of $MgSO_4.7H_2O$, K_2HPO_4, KNO_3 and $NaHCO_3$ were 0.409 g/L, 0.24 g/L, 1.033 g/L, and 3.265 g/L, respectively. The study reported that the biomass productivity of *C. pyrenoidosa* improved from 0.14 g/L in defined fog's medium to 1.40 g/L in modified fog's medium resulting 10 fold increase. The biochemical composition biosynthesis of *C. pyrenoidosa* was altered using nitrogen limiting stress bringing about 5.23 fold increase in lipid content than control (cell without stress), as analyzed by FTIR integration method.

***Keywords*—**Autotrophic condition, *Chlorella pyrenoidosa,* FTIR, Response Surface Methodology, Optimization.

I. Introduction

THE sustainable and economic feedstock, microalgal biomass is needed for the world to overcome food security problems and address environmental topics [1]. Inferring from research over the past years, it has been proved that microalgae can be utilized to produce numerous practical as well as economical vital products [2], [3]. They have appreciable growth rate, provide lipid in addition to carbohydrates for biofuel production, despite the fact that the rapid gains in the biomass production by cost effective methods are crucial [4]-[8]. Hence a successful biorefinery concept could be established via cultivation of microalgae for simultaneous production of biofuels along with high-value compounds. Furthermore, these strategies may help in the improvement of economic feasibility of the biofuel production [9]-[13]. Mode of cultivation influences the specific growth rates and biomass yield of microalgae considerably and its trophic mode is essential to enhance biomass productivity which can be regulated in unsterilized autotrophic cultivation mode [14], [15]. However, the constantly budding algae with increasing cell density diminishes the light penetration that limits growth to a final cell concentration which rarely exceeds 0.5 g/L on dry weight basis [16]. Mainly nutritional and environmental components are very important for the optimization of cultivation parameters [17], [18]. The statistical methods are supposed to be the efficient and potent approaches meant for screening main elements of a multivariable framework to improve medium conditions and have been widely used in recent reports [19]-[22]. Several studies reported the cultivation of *C. pyrenoidosa* in media containing various carbon sources such as glucose, acetate, sucrose, glycerol and wastewaters such as poultry litter extract and carpet industry wastewater [23]-[25], though no special consideration has been paid to statistical look over the autotrophic medium components of *C. pyrenoidosa* for biomass generation. Since media optimization using a one-factor at a time does not involve the interactions among parameters, statistical central composite experimental design by a response surface methodology (RSM) has been applied to media optimization for biomass production [26]-[31]. Various reports showed that cultivation of microalgae in a stress environment of nitrogen (N) limited medium triggers to lipid accumulation [32]-[34] as investigated by Fourier transform infrared spectroscopy (FTIR). FTIR is a strategy used for entire cell investigation utilizing dried biomass, which includes the estimation of infrared absorption in connection to molecular vibrational modes [35]. Macromolecules (lipids, proteins, nucleic acids and carbohydrates) can be quantified and identified by specific molecular groups by their absorption bands [36]. Previous reports demonstrated the potential ability of FTIR as a tool to quantify biochemical elements, together with lipids, in response to a nutrient stress, such as low nitrogen [37]-[39] and low phosphorus [40], [41]. The aim of the present study was to evaluate the effect of individual medium components as well as the mutual interactions of the most significant components by statistical modeling on biomass production of *C. pyrenoidosa* and to evaluate the effect of nitrogen starvation on composition of *C. pyrenoidos*a biomass.

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II. Materials and Methods

A. Microorganism and Medium

Microalgal strain of *Chlorella pyrenoidosa* was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, (NCL), Pune, India. This was maintained and cultivated in a Fog's medium having the following composition (mg/L): $MgSO_4.7H_2O$ (200), K_2HPO_4 (200), $CaCl_2.H_2O$ (100), Fe-EDTA solution (5 mL) prepared by adding 0.00745g/L Na_2EDTA and 0.00557 g/L $FeSO_4.7H_2O$; H_3BO_3 (286), $MNCl_2.4H_2O$ (181), $ZnSO_4.7H_2O$ (22), $Na_2MoO_4.2H_2O$ (39), $CuSO_4.5H_2O$ (8), KNO_3 (200), pH 7.5 [42].

B. Growth Conditions and Analysis

Sterilized medium was inoculated with 5% v/v exponentially growing culture of *C. pyrenoidosa*, having viable count of 1×10^7 cells/mL. Experiment was carried out under autotrophic cultivation condition in a 250-mL Erlenmeyer flask containing 150 mL Fog's medium exposed to light intensity of 480 Wm^{-2} in 12:12 circadian cycles at 28°C for 12 days in the static conditions. The dry biomass and cellular composition of cells were then measured. All of the experiments were carried out in triplicate.

Cell concentration was determined by withdrawing 5ml of algal suspension via sampling tube and measuring the optical density at 686nm by a spectrophotometer. Biomass yield was related to optical density by the using the following equation (1) for *C. pyrenoidosa.*

$$\text{Biomass} = \frac{\text{Observed optical density}}{0.0563} \quad (R^2=0.9989) \qquad (1)$$

C. Statistical Analysis of Data

The software package, Design-Expert trial version 8.0 (Stat Ease, Inc, Minneapolis, USA) was employed to design the experiments and to analyze the results. Multiple linear regression analysis was carried out to estimate t-values, p-values to evaluate the significance of experimental design and screen out the factors affecting biomass production.

D. Statistical Optimization of the Fog's Media Components by Response Surface Methodology

The production of algal biomass is influenced by many factors including medium components. The statistical optimization of fog's medium components was done by applying central composite design (CCD), where four nutritional factors were selected and their interactive effects as well as optimum levels were determined. This optimization was attempted with a view to enhance the biomass productivity of *C. pyrenoidosa* so that can be used in biorefinery. Based upon our preliminary studies and literature review for the cultivation of *C. pyrenoidosa*, four medium components including $MgSO_4$, K_2HPO_4, KNO_3 and $NaHCO_3$ were chosen to investigate the first and higher-order main effects of each factor and interactions amongst them for further optimization of their concentrations favoring maximum biomass through response surface methodology. A 2^4 factorial central composite experimental design resulting in 30 experimental runs was generated by Design Expert, Version 8.0 (Table I). The range and levels of the variables investigated are given in Table II. The relation between coded and actual values was described according to following equation:

$$xi = (Xi - X0i)/\Delta Xi \qquad (2)$$
$$i = 1,2,3\ldots j$$

where xi = coded (dimensionless) value of the variable Xi; Xi = actual value of the i^{th} variable; X_0 = the value of Xi at the center point; ΔX = the step change value.

The behavior of the system was explained by a second order polynomial using the following equation:

$$Y = bo + \sum bi\, xi + \sum\sum bij\, xi\, xj + \sum bii\, x2i + e \qquad (3)$$

where Y= measured response; bo, bi, bij, bii are constants and regression coefficients of model; xi and xj are levels (codes values) of independent variables; e is random error. The Design Expert was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. Contour graphs were also obtained by using Design Expert software to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by the coefficient of determination (R^2). The statistical significance of model coefficient was evaluated by ANOVA.

E. Scale up of Cultivation of C. pyrenoidosa

The scale up of cultivation was performed in the optimized medium taken in 100 L glass chamber (46cm×76cm ×30cm), used as an indigenous photobioreactor with a working volume of 70 L. This was fitted with a stirrer and was illuminated with 6 compact fluorescent lamps each of 10-watt placed at a distance of 10 cm on all the sides providing 480 Wm^{-2} light intensity. The medium was inoculated with 5% v/v exponentially growing culture and incubated at 28±2°C, with agitation at 150 rpm for 12 days in 12:12 circadian cycles.

F. Biochemical Manipulation of C. pyrenoidosa Cells by Cultivation under N Starvation

The cells cultivated in autotrophic Fog's medium with initial KNO_3 content of 1600mg/L were harvested with centrifugation (5000 rpm for 10 min) and the concentrated microalgal cell pellet was washed twice with distilled water. This was then dispensed in N starved Fog's medium having initial KNO_3 of 0.8mg/L. Samples of the biomass were withdrawn after 2 and 4 days of incubation, separately under light and dark conditions.

G. Cellular Composition Analysis

FTIR spectroscopy of the algal cells cultivated in N-enriched and N-starved media was carried out to determine the chemical composition of algal biomass. PerkinElmer FTIR instrument (Central Instrumentation Laboratory, Panjab University, Chandigarh, India) was used to process the FTIR spectra ranging from 400 to 4000 cm^{-1}. The characteristic peak

areas of lipids, proteins and carbohydrates were calculated by integration using essential FTIR v3.10.004 software-Trial version, from Operant LLC. The amounts of biomolecules and their peak areas were correlated as the followings equations as in [36].

$$A_L = -2.30 + 78.96 \times T_L \quad (4)$$
$$A_P = -0.27 + 12.72 \times T_P \quad (5)$$
$$A_C = 0.07 + 2.05 \times T_C \quad (6)$$

where T_L, T_P and T_C represent the total amounts of lipids, proteins and carbohydrates in mg, and A_L, A_P and A_C are characteristic peak areas of lipids, proteins and carbohydrates, respectively.

III. Results

A. Optimization of Nutrient Sources for Biomass Production by C. pyrenoidosa Using Response Surface Methodology

To determine the optimum response regions for biomass yield, central composite design (CCD) was created with a set of four independent variables, designated as A, B, C and D (Table I) to study the combined effect of each independent variable. Each variable was studied at five different levels (-2,-1, 0,+1 and +2) as shown in Table II and their respected responses (Y) as biomass yield are depicted in Table I. To decide about the adequacy of model, two different tests viz., Sequential model Sum of Squares and Model Summary Statistics were carried out. On the basis of their p value, R^2, standard deviation and predicted sum of square (PRESS) values, the adequacy of the quadratic regression model was found to be significant for biomass production. The statistical significance of the ratio of mean square variation due to regression and mean square residual error was tested using the analysis of variance (ANOVA) which is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters of the model [43]. The associated p-value was used to estimate whether F value is large enough to indicate statistical significance. If p-value is lower than 0.05, and then it indicates that the model is statistically significant [44].

The ANOVA result of the biomass production shows the quadratic model with F-value of 91.30 and p-value <0.005 to be significant (Table III). Of the various regression coefficients, $MgSO_4$ (A) and $NaHCO_3$ (D) were insignificant in the model owing to their p-values >0.005. The goodness of the fit of the model was checked by the determination of correlation coefficient (R^2) which was calculated to be 0.9847, indicating that 98.47 % of variables fit the response. The "Pred R-Squared" of 0.9416 is in reasonable agreement with the "Adj R-Squared" of 0.9739. The value signal to noise ratio (*S/N)*, which is a measure of the adequate precision is 33.193. A value greater than 4 is desirable in support of the fitness of the model [45]. This thus confirms that the model is statistically sound and can be used to navigate the design space.

The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower is the reliability of experiment. In this experiment, a lower value of CV %, corresponding to 1.82 indicates a greater reliability of the experiments performed. The analysis shows that the form of the model chosen to explain the relationship between the factors and the response is correct.

The ANOVA analysis indicates a linear relationship between the significant effects of A ($MgSO_4$), B (K_2HPO_4), C (KNO_3) and D ($NaHCO_3$) the interaction between $MgSO_4$ and K_2HPO_4, $MgSO_4$ and $NaHCO_3$, K_2HPO_4 and KNO_3, KNO_3 and $NaHCO_3$, the quadratic relationship with $MgSO_4$, K_2HPO_4, KNO_3 and NaHCO3. By applying multiple regression analysis on the experimental data, second order polynomial equation (7) was found to calculate the biomass production by considering the various terms.

$$\text{Biomass} = +1.16 - 0.000998 \times A + 0.053 \times B - 0.041 \times C - 0.00377 \times D - 0.057 \times A \times B - 0.076 \times A \times D + 0.035 \times B \times C - 0.049 \times C \times D + 0.018 \times A^2 - 0.032 \times B^2 + 0.031 \times C^2 - 0.051 \times D^2 \quad (7)$$

TABLE I
CENTRAL COMPOSITE DESIGN MATRIX WITH EXPERIMENTAL VALUES OF TOTAL BIOMASS PRODUCTION BY *CHLORELLA PYRENOIDOSA*

Runs	A: $MgSO_4$	B: K_2HPO_4	C: KNO_3	D: $NaHCO_3$	Response (Y): Biomass (g/l)
1	+1	-1	+1	+1	0.92
2	+1	-1	+1	-1	1.16
3	0	0	0	-2	0.96
4	0	0	0	0	1.15
5	0	0	0	0	1.15
6	-1	+1	+1	-1	1.22
7	0	+2	0	0	1.14
8	+1	-1	-1	-1	1.26
9	+1	+1	+1	+1	0.98
10	-1	-1	+1	-1	0.95
11	0	0	0	0	1.17
12	0	0	0	0	1.16
13	-2	0	0	0	1.22
14	0	0	-2	0	1.39
15	-1	-1	-1	+1	1.21
16	+1	+1	-1	-1	1.14
17	+2	0	0	0	1.25
18	-1	+1	-1	+1	1.36
19	0	0	0	+2	0.96
20	0	-2	0	0	0.92
21	0	0	0	0	1.16
22	-1	-1	+1	+1	0.96
23	+1	+1	-1	+1	1.09
24	-1	-1	-1	-1	0.94
25	+1	-1	-1	+1	1.14
26	0	0	0	0	1.15
27	+1	+1	+1	-1	1.22
28	-1	+1	-1	-1	1.10
29	0	0	+2	0	1.19
30	-1	+1	+1	+1	1.25

TABLE II
LEVELS OF INDEPENDENT VARIABLES IN RESPONSE SURFACE METHODOLOGY

Variables	Coded levels				
	-2	-1	0	+1	+2
A: $MgSO_4$ (g/L)	0.1	0.2	0.3	0.4	0.5
B:K_2HPO_4 (g/L)	0.1	0.2	0.3	0.4	0.5
C:KNO_3 (g/L)	1	2	3	4	5
D:$NaHCO_3$ (g/L)	1	2	3	4	5

B. Interactions among the Factors

Student's t–test was employed to determine the knowledge of the error mean square that is essential in testing the significance of the estimated coefficient of the regression equation. The larger magnitude of t value and smaller p value makes the corresponding coefficient more significant [46]. Coefficient estimates and t-values in the quadratic model as depicted in Table II indicate that factor B (K_2HPO_4) had positive effect on biomass yield. The interactions between the factors AB, AD and CD showed negative effects on biomass yields while the interactions between BC had a positive effect. Fig. 1 depicts the contour graphs revealing the interactions between two factors for the optimization of conditions for biomass production. From the plots, it was easy and convenient to understand the interactions between two variables and to locate the optimum levels. Each curve represents an infinite number of combinations of two test variables with the other variables maintained at a constant level. The contour graph obtained as a function of $MgSO_4$ concentration versus K_2HPO_4 concentration indicated that biomass production increased with the increase of both $MgSO_4$ and K_2HPO_4. The interactive effect of both the parameters indicated that the production increased gradually with the increase in levels of KH_2PO_4 with minimum MgSO4. The maximum production of biomass corresponding to 1.42 g/L was obtained in the Fog's medium where the concentrations of $MgSO_4$ and K_2HPO_4 were 0.10g/L and 0.50 g/L respectively (Fig. 1 (a)) while KNO_3 and $NaHCO_3$ was held at 0, 0 coded level. The contour graph obtained as a function of $MgSO_4$ concentration versus $NaHCO_3$ concentration showed that the biomass productivity increased with the concentration of both $MgSO_4$ and NaHCO3. The interactive effect of both the parameters indicated that the production increased gradually with the increase in level of $MgSO_4$ from minimum to maximum while with $NaHCO_3$ it increased up to 1.61 g/L and decreased thereafter. The maximum biomass productivity of 1.34 g/L occurred at a concentration of 0.50 g/L of $MgSO_4$ and 1.61g/L of $NaHCO_3$ with K_2HPO_4 and KNO_3 held at 0, 0 coded levels respectively (Fig. 1 (b)). Fig. 1 (c) shows the effect of K_2HPO_4 and KNO_3 on biomass production.

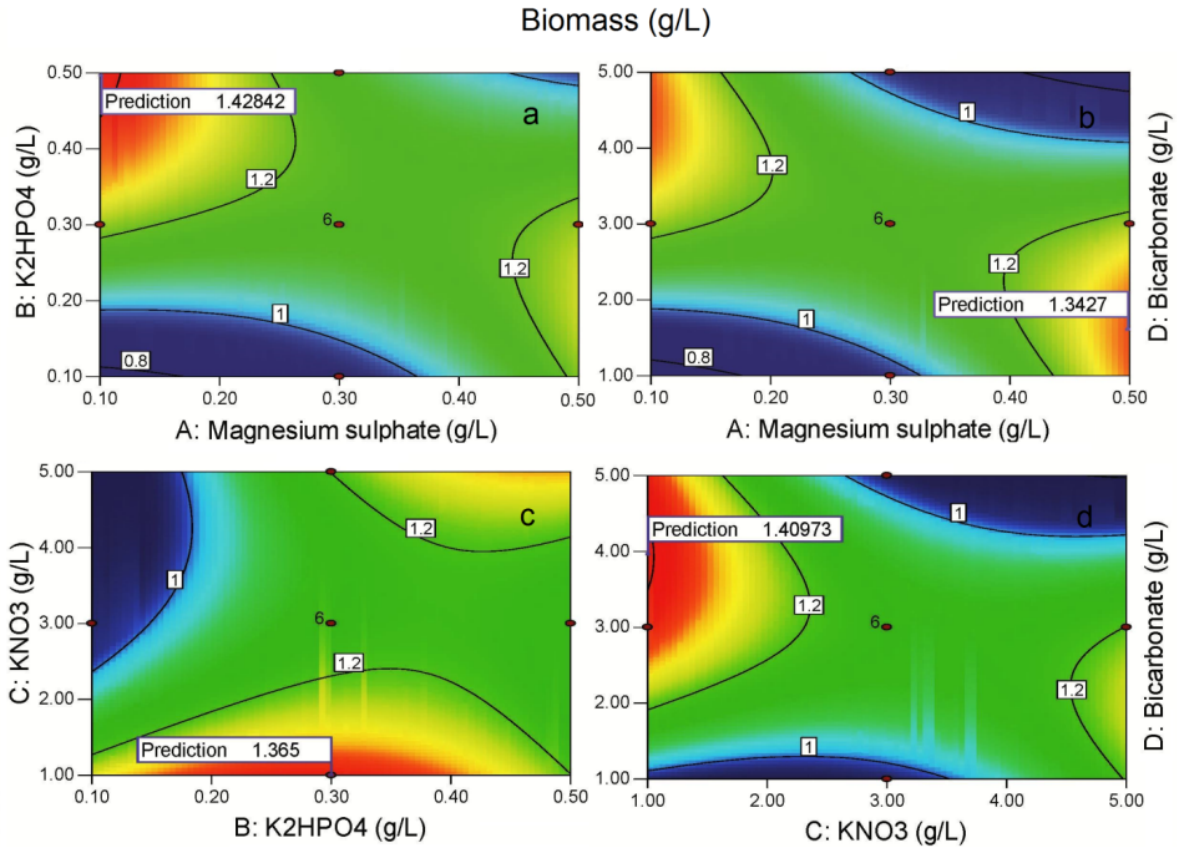

Fig. 1 Contour plots representing biomass yield from culture of *C. pyrenoidosa* as affected by cultural conditions (a) K_2HPO_4 and $MgSO_4$ (b) $NaHCO_3$ and $MgSO_4$ (c) KNO_3 and K_2HPO_4 (d) $NaHCO_3$ and KNO_3. All the values expressed in terms of g/L.

TABLE III
ANOVA RESULTS FOR BIOMASS PRODUCTION UNDER RESPONSE SURFACE QUADRATIC MODEL AND MODEL COEFFICIENTS ESTIMATED BY MULTIPLE LINEAR REGRESSION

Variables	Sum of squares	Coefficients	t-test	F-value	p-value	Confidence Level (%)
Model	0.465029	1.156878	137.5693	91.3	< 0.0001	99.99
A:	0.0000239	-0.001	-0.23723	0.1	0.8153	18.47
B:	0.066238	0.052535	12.49431	156.1	< 0.0001	99.99
C:	0.041238	-0.04145	-9.85838	7.2	< 0.0001	99.99
D:	0.000341	-0.00377	-0.89622	0.8	0.3827	61.73
AB	0.051724	-0.05686	-11.0409	121.9	< 0.0001	99.99
AD	0.019875	-0.07598	-14.7535	217.7	< 0.0001	99.99
BC	0.037965	0.035245	6.844088	46.8	< 0.0001	99.99
CD	0.02863	-0.04871	-9.45905	89.5	< 0.0001	99.99
A^2	0.026699	0.018398	4.677762	21.9	0.0002162	99.97
B^2	0.071605	-0.03231	-8.21426	67.5	< 0.0001	99.99
C^2	0.465029	0.0312	7.932471	62.9	< 0.0001	99.99
D^2	0.07161	-0.05109	-12.9907	168.8	< 0.0001	99.99

STD.DEV. = 0.021, R^2 = 0.9847, MEAN = 1.13, ADJ R^2 = 0.9739, PRED. R^2 = 0.9416, C.V. % = 1.82, PRESS = 0.028, ADEQ PRECISION = 33.193, A= $MgSO_4$, B= K2HPO4, C= KNO_3, D= $NaHCO_3$.

Increase in the concentration of K_2HPO_4 promoted the biomass production up to a concentration of 0.3 g/L and decreased with further increase in its concentration. On the other hand the biomass production decreased with the gradual increase in KNO_3 level. The maximum production of biomass corresponding to 1.365 g/L was obtained at 0.3 g/L and 1.0 g/L of K_2HPO_4 and KNO_3 respectively, with $MgSO_4$ and $NaHCO_3$ held at 0 coded levels. Fig 1 (d) shows the effect of KNO_3 and $NaHCO_3$ on biomass production which increased gradually with the concentration of $NaHCO_3$ upto 3.96 g/L. On the other hand the yield decreased with the concentration of KNO_3. The maximum biomass productivity of 1.409 g/L was obtained at 1.0 g/L and 3.96 g/L concentrations of KNO_3 and $NaHCO_3$ respectively, with K_2HPO_4 and $MgSO_4$ held at 0 coded levels.

C. Model Validation

In order to evaluate the accuracy of statistical experimental model of Response Surface Methodology (RSM), attempts were made to formulate a medium for maximizing the biomass yield by using different variables. Numerical optimization for biomass production was attempted with the software package, Design-Expert trial version 8.0 (Stat Ease, Inc, Minneapolis, USA) using the variables A ($MgSO_4$; 0.409 g/L), B (K_2HPO_4; 0.249 g/L), C (KNO_3; 1.033 g/L) and D (NaHCO3; 3.265 g/L) in Fog's medium, predicted the biomass yield of 1.41 g/L. To validate the optimum concentrations of the variables, an experiment was performed using the medium with the above specified conditions, inoculated with 5% (v/v) of culture of *Chlorella pyrenoidosa,* incubated at 28°C under 12:12 h light-dark period in a stationary state for 12 days and the biomass yield was 1.40 g/L which is 0.71% less than the predicted value. This high degree of accuracy obtained confirms the validity of the model with minor discrepancy due to the slight variation in experimental conditions. Statistical evaluation of culture conditions thus enhanced the production of biomass to an appreciable amount.

D. Scale up of Cultivation

Open tanks with different sizes of transparent rectangular chambers have been used for cultivation of microalgae [47] and the important physical (light; temperature; mixing intensity), chemical (dissolved CO_2 and oxygen; pH; and nitrogen), and biological (biomass concentration and biomass composition) parameters have been monitored and regulated. *C. pyrenoidosa* grown in a 100 L photobioreactor with working volume of 70 L as shown in Fig. 2 illuminated with 6 CFL and mixing intensity of 150 rpm also revealed a biomass yield of 1.0 g/L after 12 day of incubation with 12:12 circadian cycles.

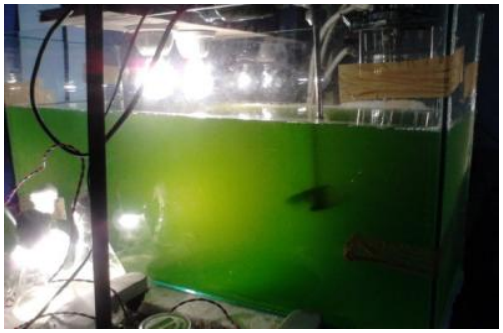

Fig. 2 Open glass chamber used for large scale production of biomass

E. Consequences of N Starvation on Cellular Composition of Algae

Many researchers have reported the significant effect of stress factors like iron, copper, chloramphenicol, nutrient starvation and light-dark condition on the growth and cellular variability in microalgae [48]-[51]. The microalgae, *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* were evaluated by FTIR in terms of lipid and carbohydrate content which was cultivated in limiting concentrations of N [52]. Following N starvation in the Fog's medium, *C. pyrenoidosa* showed both increased cell size and limited cell division. This is possibly due to increased adenosine mono phosphate deaminase (AMPD) content of the cells under N

starvation which leads to the catalysis of adenosine monophosphate (AMP) to inosine monophosphate (IMP) and ammonia. Decrease in AMP would partially or completely inhibit the isocitrate dehydrogenase activity as this is AMP dependent [53]. Giordano et al. [37] reported the trend of crude protein content variations with respect to N concentration in culture medium in *Scenedesmus* sp. CCNM 1077. It was found that total N removal from culture medium decreased the crude protein content and re-addition of N in culture medium showed an increased protein content in microalga.

The effect of N starvation on cellular components like lipid, crude protein and carbohydrate in *C. pyrenoidosa,* as observed in the present study, is represented in Fig. 3. The decrease of N concentration in the culture medium from 1600 to 0 mg/L resulted in an increase in protein content to 80±1% in N starved condition in the presence of light, which was about 26.9% higher than the N rich culture but it was found that in the absence of light, crude protein content decreased to 29±2% which was about 54% and 64% less than those present in biomass cultured N rich and N starved condition in the presence of light respectively. A possible reason of decrease in protein in N starved dark cultivation strategy is that the cells may have tarnished the nitrogenous compounds to sustain intracellular protein part in support of their regular metabolic function. Further, when photosynthetic microalgae cultivated under N starved condition, carbon flow from photosynthetic pathways convert into various modes to channel metabolic energy into different energy rich components like lipids and carbohydrate [54]. The algal storage products (lipid and carbohydrate) are preferred under stress probably for use during adverse conditions for cell survival and division. These are in highly reduced states and being hydrophobic in nature can be bundled in a small chamber of cell [55]. Fig. 3 shows that *C. pyrenoidosa* cultivated in N starved medium significantly increased the lipid content from 13% in N rich condition to 63±5% after 4 days of cultivation in N deficient conditions in dark.

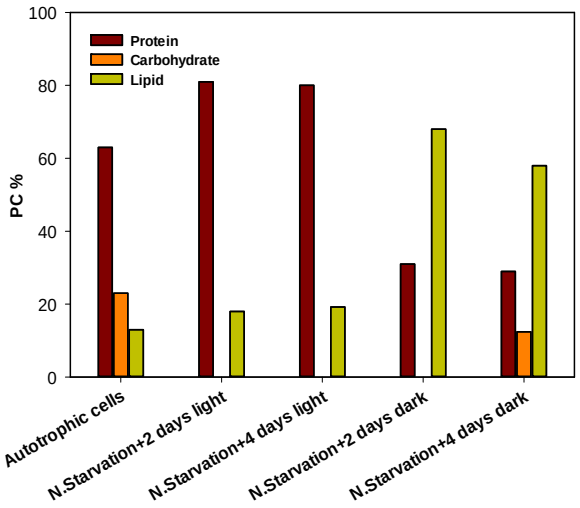

Fig. 3 The cellular variability of *C. pyrenoidosa* in terms of protein, carbohydrate and lipid content under N stress conditions on day 2 and day 4. PC refers to Principal Components derived from the data analysis of peak area integration from FTIR

Table IV represents the changing spectral ratios due to the application of N starved condition. The study also revealed that under N starved cultivation the cells had negative co-relation with the cellular carbohydrate and lipid content. *C. pyrenoidosa* cells grown in N starved dark medium resulted in an increase in lipid and decrease in protein content, whereas in the N starved medium exposed to light, it showed a significant increase in protein whereas no significant increase in lipid content were found.

Cells under N starved light exposure for 2 days showed the maximum lipid/Amide II. The study also indicated that 4 day light exposure to N-starved culture had slightly less amount of lipid as observed in 2 day exposed culture while the protein content almost remained the same. However, there was no accumulation of total carbohydrate indicating that *C. pyrenoidosa* is probably a lipid accumulating microalga under N-stress. Total removal of N from the culture medium resulted in highest lipid (68%) and protein content (81%), making it a potential feedstock for biodiesel and value added food supplement as a single cell production.

TABLE IV
THE CHARACTERISTIC PEAK AREAS OF LIPIDS, PROTEINS AND CARBOHYDRATES RESULTED FROM ABSORBANCE BAND OF FTIR SPECTRA FROM DRIED ALGAL SAMPLE

Different stress conditions	Lipid[a]	Protein[b] (AmideII)	Carbohydrate[c]	Lipid/AmideII[b]	Carbohydrate/ AmideII[b]
Autotrophic cells	0.2853	1.7227	0.1906	0.165612	0.11064
N.Starvation+2days light	0.3131	1.5839	-0.4680	0.197677	-0.29547**
N.Starvation+4days light	0.3051	1.4426	-0.4618	0.211493*	-0.32012
N.Starvation+2days dark	0.9432	5.3889	-0.1349	0.175026	-0.02503
N.Starvation+4days dark	0.4745	2.9462	0.1417	0.161055	0.048096

*maximum lipid content, ** (-) shows absence of carbohydrate content, corresponding wave number ranges: a-2,984–2,780 cm^{-1}, b- 1,590 and 1,477 cm^{-1}, c-1,180 and 1,133 cm^{-1}. The amide II band is best used for quantitative estimation of protein content [36]

IV. Conclusion

Chlorella pyrenoidosa could grow optimally in the autotrophic culture medium containing 0.409 g/L, 0.249 g/L, 1.033 g/L and 3.265 g/L, of $MgSO_4.7H_2O$, K_2HPO_4, KNO_3 and $NaHCO_3$ respectively. The maximum growth of *C. pyrenoidosa* in modified fog's medium was about 10 fold higher than under fog's medium. Further, the nitrogen starvation revealed a 5.23 fold increase in lipid content. The outcome of this study can be practiced within aquacultures for the improved algal biomass production and manipulation of its biochemical composition without metabolic engineering.

Acknowledgment

The financial assistance provided by University Grant Commission (UGC) in the form of Research Fellowship to Mrs. Nisha Phour Dhull is highly acknowledged. The authors are thankful to NCL, Pune for providing the algal sample.

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