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Statistical analysis on stress induced lipid accumulation along with the major cell components of *Chlorella* sp.

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Abstract: A sequential optimization based on statistical design was employed to optimize the different stress conditions of autotrophic, heterotrophic and mixotrophic levels for the enrichment of lipid in fresh water algae *Chlorella* sp. The selected individual parameters such as initial pH level, glucose concentration and KNO_3 concentration of the medium were optimized by the central composite design under response surface methodology. The maximum lipid content of 49.8% was achieved at the mixotrophic growth condition at 5 g/L of glucose concentration, 3 g/L of KNO_3 concentration and pH 7.6 amidst the considered different growth conditions such as autotrophic, heterotrophic and mixotrophic levels comprising along with that of the different individual stress parameters. Similarly maximum protein and chlorophyll yields were achieved at the mixotrophic level with high nitrogen content.

Keywords: Algae, *Chlorella* sp., Glucose, pH, Concentration.

Introduction

The need to fulfil the ever increasing global energy demand causes the intensive use of fossil fuels like coal, petroleum and natural gas during the last century. Nowadays they represent more than 80% of the energy resources. Owing to the fact about their exhaustibility and potential environmental impact generated by their fossil origin, growing attention about the renewable energy resources have emerged, with the aim of diversifying the energy resources to reduce the utilization of fossil fuels and hence, limiting their negative effect. In this context one of the most important renewable energy resource is biodiesel, which is produced from triglycerides by transesterification reactions[1].Commercial production of microalgae has been practiced since 1950, commencing with the production of *Chlorella vulgaris* in Japan and Taiwan, and later with the production of *Spirulina*. The past decade had witnessed great strides in the production and utilization of algae

Today, biomass covers about 10% of the world's primary energy demand. Against a backdrop of rising crude oil prices, depletion of resources, political instability in producing countries and environmental challenges, besides the efficiency and intelligent use, only biomass has the potential to replace the supply of an energy ravenous civilization. Actually, one of the most promising feedstock for biodiesel production is unicellular algae[3]. In fact, when compared with superior plants, microalgae show high photosynthetic efficiency, higher biomass productivities and faster growth rates and microalgae are very diverse in nature[4-5]. They can create a range of useful products, and there is a variety of ways in which they can be cultivated, manipulated, harvested and utilized[6]. The most important reason for this study is microalgae have the ability to produce large amounts of lipids, including triacylglycerides (TAGs), a high energy density storage molecule[7],

which can be converted into biodiesel by means of transesterification. Natural oil levels vary between microalgae species; a review of 14 microalgae genera reported oil contents of 15-77% total dry weight[8]. Lipid production can be increased by manipulating the environment of the microalgae, also known as biochemical engineering[7]. As lipid content is affected by many environmental factors, there exists a variety of ways of doing this in different algal species, including nitrogen deprivation, temperature and light, pH stress, CO_2 aeration and osmotic stress[9-10]. It has been reported that biodiesel obtained from canola and soybean, palm, sunflower oil, algal oil acts as a diesel fuel substitute[11]. Some algal species are halo tolerant and they produce lipids as compatible solutes to cope with high or fluctuating salinities. Proteins make up a large fraction of the biomass of actively growing microalgae and cyanobacteria, although they are generally undervalued compared to minor products such as omega fatty acids[12].

It has been estimated that at low nitrogen concentrations, *Chlorella* synthesized gets saturated (16:0) and monounsaturated (18:0) fatty acids are responsible for higher biodiesel yield. In most of the *Chlorella* sp. reduced nitrogen content in the medium, increases the lipid content[13]. It is also said that the nitrate deficiency doesn't inhibit the logarithmic growth rate until the culture reaches nitrogen deprivation. Nitrogen source and concentration in the growth media greatly influence the yield of algae lipid. In nitrogen-limited situations, algae lipid content usually increases because lipid-synthesizing enzymes are less vulnerable to incompetence than carbohydrate synthesizing enzymes due to nitrogen deprivation; thus the major proportion of carbon can be bound in lipids[14].

Chlorella vulgaris has a greater potential as a resource for the production of biodiesel due to faster growth and easier cultivation. However, lipid content in *Chlorella vulgaris* under general growth conditions are approximately 20% by weight of dry biomass[13], which cannot meet the standard industrial requirements. In this study, we deal with an in-depth investigation of the growth rate and lipid yield of *Chlorella* sp. Since a wide range of growing conditions are present. Combinations of starvations were designed to turn the metabolism into an anabolic lipid accumulating phase. In order to obtain matchable data, the assays were conducted in the same conditions of irradiance and algal concentration in regimen of autotrophy, mixotrophy and heterotrophy by performing response surface methodology by considering three parameters such as glucose concentration, potassium nitrate concentration and pH of the medium. The effect of these varying conditions on algae with respect to protein, chlorophyll and lipid content were recorded. Objective of the study is to define the most suitable growing conditions for higher lipid accumulation.

Materials and Methods

Microorganism, growth conditions and inoculum preparation

The micro algae *Chlorella* sp. was isolated from fresh green water of local pond under serial dilution and periodic sub culturing. Its identification was done by microscopic examination. It was raised in Bold Basal Medium (BBM). The microalgae, *Chlorella* sp. was inoculated in BBM incorporated with 30 g/L of glucose and 4 g/L of yeast with a pH of 6.6 and the culture was incubated for 4 days at 24±1ºC in a thermo-statically controlled chamber and illuminated with cool inflorescence lamps at an intensity of 60 µmol/ (m^2 s) (14 WTL5 tungsten filament lamps; Philips Co.).

Optimization of stress condition

The central composite design (CCD) under the response surface methodology (RSM) was employed in order to illustrate the nature of the response surface in the experimental design and elucidate the optimal conditions of the most significant independent variables. In this analysis initial pH, glucose concentration (g/L) and KNO_3 (g/L) concentration were taken as independent variables and the lipid yield, protein content and chlorophyll content were obtained as dependent output response variables (Table- 1).

Table 1: Experimental range and levels of independent variables

Independent variables	Design variables	Range and levels		
		-1	**0**	**1**
Glucose	A	0	5	10
KNO_3	B	0	3	6
pH	C	5.6	6.6	7.6

In order to study the combined effects of these variables depending on the responses, 20 sets of experiments with appropriate combinations of initial pH, glucose concentration and KNO_3 concentration were

conducted using statistical method. The first independent variable (initial pH) was varied over 2 levels (5.6 and 7.6) relative to the centre point (pH 6.6), the second independent variable (glucose concentration) was varied over two levels (0 g/L and 10 g/L) relative to the centre point (5 g/L) and finally the third independent variable (KNO_3 concentration) was changed over two levels (0 g/L and 6 g/L) relative to the centre point 3 g/L. The numerical analysis for the responses of lipid yield, protein and chlorophyll content was estimated using the software Minitab 14.

The evaluation of the efficiency to fit the model was done through coefficient determination and analysis of variances. The experimental results were fitted to a second order polynomial equation;

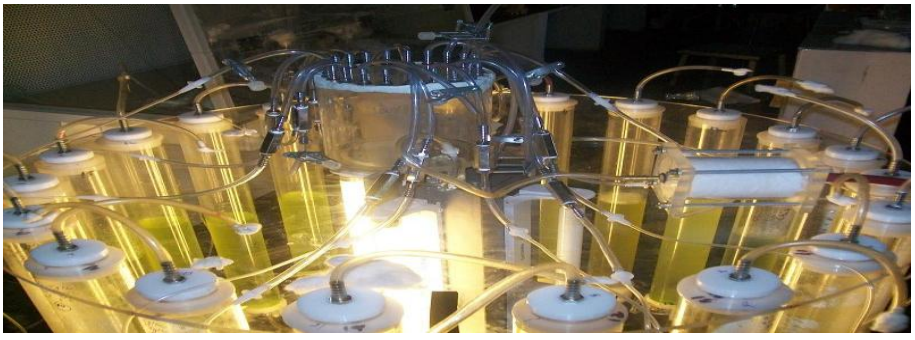
$$Y = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where, Y is the dependent variable (lipid yield, protein content and chlorophyll content); A, B and C are the independent variable; β_0 is the regression coefficient at center point; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the second order interaction coefficient. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), p- and F-values. Their ability to fit the polynomial model equation was expressed by the coefficient by determination of R^2. The statistical software package was used in order to identify the experimental design as well as to generate a regression model to predict the optimum combinations considering the effects of linear, quadratic and interaction of the three dependant responses. A final experiment was conducted to validate the CCD model developed.

Experimental design

The photo bioreactor setup required for operating the above discussed independent variables consists of 20 tubular reactors with one tube in excess considered as reference (Figure- 1). The reactor was subjected to illumination by the providing an illumination from the centre by means of four inflorescence lamps at an intensity of 60 µmol/ (m^2s) (14 WTL5 tungsten filament lamps; Philips Co.,) with the supply of CO_2/ air from the bottom as per the medium requirement at a low flow rate of 0.003 vvm using check and control valves.

Figure 1: Experimental set up of algal photo bioreactor



Cultivation in the photo bioreactor

The sterilized culture medium for all the reactors were prepared according to their run and aseptically transferred into the individual reactor, in excess and one reactor was maintained by photoheterophic, subjected to above condition to measure their deviation in experimental parameters. Photoheterotrophic cultures of *Chlorella* sp. in logarithmic phase were inoculated into the culture medium. All the dependent variables were estimated with respect to the maximum lipid yield with the minimum time period. The reactor was subjected to continuous illumination.

Estimation of major cell components

The spectral analysis was carried out (540 nm) for monitoring the cell growth using ELICO SL 150. The biomass concentration was recorded by collecting each day sample centrifuged at 14,000 ×g for 10 min. The cell pellets were lyophilized to constant weight and it was measured in terms of dry weight. In order to estimate the cellular lipids, the lyophilized cells were saponified to hydrolyse the ester bonds of membrane lipids, triglycerides and were estimated by using rapid colorimetric determination[16] using lauric acid equivalent as a standard. In order to estimate the protein content, the required disrupted cell mass samples were subjected Lowry's method[17] and the total chlorophyll content was determined spectrophotometrically[18-19].

Preparation of the fatty acid methyl esters

The lipid fraction was extracted from the biomass by the Bligh and Dyer method[20], obtaining an immiscible system consisting of sample water content and a mixture of chloroform. The lipid content was concentrated and estimated from the chloroform extract using rotary vacuum evaporator. The method of Hartman and Lago[21] was used to saponify and esterify the dried lipid extract to obtain the fatty acid methyl esters. The methyl esters were solubilised in *n*-heptane before introducing it in the gas chromatography.

Results and Discussion

Response surface estimation

The need to ensure the effectiveness of enrichment of lipid with growing algae has stimulated the interest to use mathematical models predicting microbial behavior. The objectives of this study are to investigate the combined effects of the stress induction factors such as pH, glucose concentration and KNO_3 concentration (independent variables). These variables which are to be optimized for the maximum yield of lipids and analyzing it with the protein, chlorophyll content (Table- 2). The experimental and the predicted values of lipid yield, protein content and chlorophyll content agreed very well when analyzed in Minitab 14.

Table 2: Experimental and predicted responses

Run	Type	Age	Glucose (g/L)	KNO_3 (g/L)	pH	Lipid (%)		Protein (%)		Chlorophyll (%)	
						Exp	Pred	Exp	Pred	Exp	Pred
1	PA	7	0	0	5.6	26.2	25.37	29.3	28.31	5.3	4.62
2	H	8	10	0	5.6	26.1	20.63	32.6	27.46	1.6	1.11
3	PA	8	0	6	5.6	20.2	22.34	33.5	31.03	8.3	7.68
4	H	9	10	6	5.6	22.5	23.65	43.7	43.03	0.4	-0.2
5	PA	8	0	0	7.6	32.3	29.39	34.8	34.51	0.2	0.71
6	H	10	10	0	7.6	33.1	29.2	26.6	28.11	4.4	4.9
7	PA	9	0	6	7.6	25.1	28.81	35	39.18	1.6	1.97
8	H	15	10	6	7.6	35.6	34.67	45.6	45.63	1.2	1.76
9	PA	8	0	3	6.6	32.8	30.68	48.6	48.18	2	2.42
10	H	14	10	3	6.6	22.1	31.24	46.7	50.98	0.5	0.56
11	M	10	5	0	6.6	20.1	33.2	31.9	36.82	2.8	2.96
12	M	15	5	6	6.6	40.5	34.42	48	46.94	2.6	2.92
13	M	9	5	3	5.6	43.3	46.3	10.8	20.08	1	3.42
14	M	11	5	3	7.6	49.8	53.82	29.9	24.48	4.4	2.46
15	M	12	5	3	6.6	46.4	44.03	40.9	39.55	2.4	2.27
16	M	12	5	3	6.6	46.3	44.03	40.8	39.55	2.5	2.27
17	M	12	5	3	6.6	46.2	44.03	40.4	39.55	2.4	2.27
18	M	12	5	3	6.6	46.2	44.03	41.1	39.55	2.5	2.27
19	M	12	5	3	6.6	46.8	44.03	40.5	39.55	2.4	2.27
20	M	12	5	3	6.6	46.3	44.03	41.3	39.55	2.4	2.27

Experiments were carried out as per the design matrix of the central composite design (CCD) and the average lipid yield, protein content and chlorophyll content (dependent variables) obtained from the culture was used as response.

$$\textit{Lipid content (\%)} = 270.638 + 3.48A + 5.16B - 77.55C - 0.523A^2 - 1.14B^2 + 6.03C^2 + 0.101AB + 0.23AC + 0.204BC \quad (2)$$

$$\textit{Protein content (\%)} = -723.8 - 2.55A - 2.01B + 230.98C + 0.401A^2 + 0.26B^2 - 17.26C^2 + 0.214AB - 0.28AC + 0.163BC \quad (3)$$

$$\textit{Chlorophyll content (\%)} = 43.81 - 2.19A + 0.91B - 10.71C - 0.031A^2 + 0.074B^2 + 0.664C^2 - 0.073AB + 0.385AC - 0.15BC \quad (4)$$

where A is pH, B is the glucose concentration (g/L) and C is the KNO_3 concentration (g/L)

ANOVA was carried out for the results of quadratic models for the lipid yield, protein content and chlorophyll content. The associated probability is greater than F value for each model (0.015 lipid content, 0.004 for protein content and 0.01 for chlorophyll content). F value was observed to be lower than 0.05 (Table- 3).

Table 3: The regression coefficient, variation and the p-value for the responses

Response	R^2	variation	p-value
Lipid	0.798	0.202	0.015
Protein	0.854	0.146	0.004
Chlorophyll	0.817	0.183	0.01

At the model level, the correlation measure for the estimation of the regression equation is the determination of the coefficient R^2. The correlation between the experimental and predicted values was better when the value of R^2 was closer to 1.0. In this experiment, the value of R^2 for lipid, protein and chlorophyll content were 0.798, 0.854 and 0.817 respectively. These values indicate a high degree of correlation between the experimental and the predicted values. The value of R^2 indicates that 79.8%, 85.4% and 81.7% of the variables namely pH, glucose concentration and KNO_3 concentration which contribute positively to the response. The value of R^2 is also considered as a measure of fit of the model and it can be mentioned that only about 20.2% of the total variations were not explained by the lipid content, 14.6% of the total variations were not explained by the protein content and 18.3% of the total variations were not explained by the chlorophyll content. Linear and quadratic effects of parameters were significant, depicting that they can act as limiting nutrient and little variation in their concentration would alter either the growth rate or the product formation rate or both in considerable extent.

The main target of response surface was to hunt efficiently for the optimum values of the variables such that the response was maximized. An elliptical response surface in the entire region was found from the second order quadratic equation for the higher lipid yield with the interaction of the independent variables. The maximum lipid yield of 49.8% was analyzed at pH 7.6, 5 g/L of glucose concentration, 3 g/L of KNO_3 concentration which were said to be the independent variables at the mixotrophic level of growth condition. Here the nitrogen content in the medium was said to be limited and hence higher accumulation of lipid yield was higher than the amount predicted by Rodolfi *et al.,* 2009[22]. At this significant condition, 29.9 % of protein content and 4.4 % of chlorophyll content were obtained. At the mixotrophic level of stress condition comprising pH 6.6, 5g/L of glucose concentration and 6 g/L of KNO_3 concentration, both the protein and chlorophyll content has marked its higher value of 48 % and 2.6 %. At this condition, the lipid content was said to be 40.5 %. This characteristic difference has helped to predict the dependence of the chosen dependent variables towards the concentration difference in the supply of the nitrogen source; based on the obtained values, the higher lipid content of 49.8% was obtained at the mixotrophic condition with limitation in the nitrogen concentration. The protein content and chlorophyll has been observed to be decreased with the decrease in the nitrogen content. Significantly it was observed that on the presence of higher amount of glucose content in the medium, stress associated with the depletion of the nitrogen content leads to the restriction of growth of biomass concentration (data not shown) to the appropriate independent variables of pH 5.6, 10 g/L of glucose concentration and 0 g/L of KNO_3 concentration. On the subsequent characteristic study associated with the comparison of different combinations of stress conditions, lead to the absolute understanding of the nature of growth and associated production of cell components of *Chlorella* sp.

Analysis of algal biodiesel conversion

For the quantification of reaction product, the algal biodiesel samples were analyzed by a gas chromatography (GC)-Perkin Elmer GC. The content of the fatty acid methyl ester in the final product was calculated quantitatively by comparing the peak areas of fatty acid methyl esters to the peak area of internal standard (lauric acid, C12:0) obtained from GC (Figure- 2 & 3).

Figure 2: GC analysis of fatty acid methyl ester with lauric acid as a internal standard

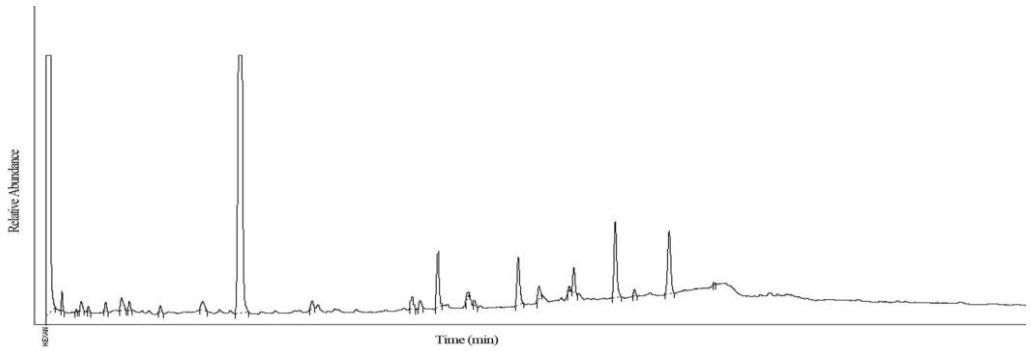
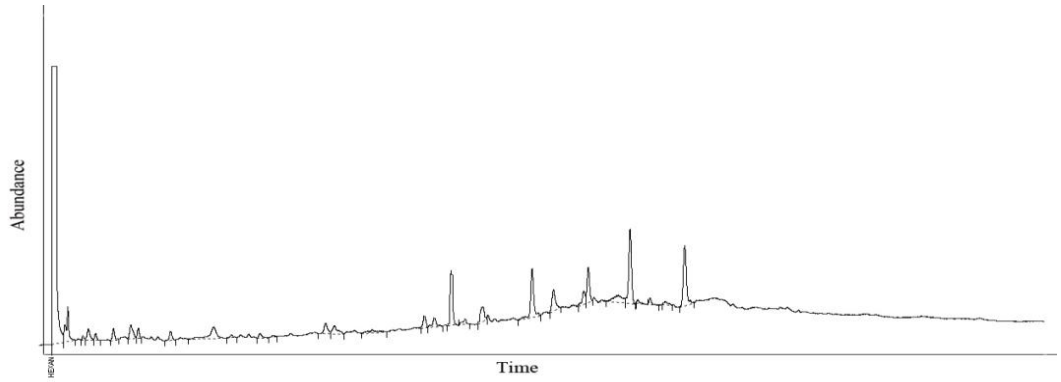


Figure 3: GC analysis of fatty acid methyl ester



It was noted that the algal biodiesel contains major proportion of saturated fatty acids, mono and poly unsaturated fatty acids (Table- 4). The major long chain fatty acids were icosanoic acid (C20; 12.66%), octadeca-9-enoic acid (C18:1, 2.166%), octadecatrienoic acid (C18, 11.67%). Our sample contains PUFA such as γ-linolenic acid (cis 6,9,12 octadecatrienoic acid) and α linolenic acid (octa decatrienoic acid). The major saturated fatty acid present is the arachnoid acid (12.66%). The total PUFA in sample was 16.69% and the major PUFA was octadecatrienoic acid (11.67%).

Table 4: Compositions of fatty acid in the sample

Peak	Time	Area (%)	Name of Fatty Acid	Type	C
1	10.23	6.09	Hexanoic acid	Saturated	5
5	11.9	1.54	octanoic acid	Saturated	8
8	13.91	1.36	Decanoic acid	Saturated	10
9	15.45	5.14	Unseanoic acid	Saturated	11
13	19.77	2.53	Tetradecanoic acid	Saturated	14
14	21.12	1.5	Tetradeca 9 enoic acid	Saturated	14
18	23.98	8.88	cis 10 pentadecanoic acid	Saturated	15
19	24.5	1.13	pentasecanoic acid	Saturated	15
20	25.11	3.56	Hexadecanoic acid	Saturated	16
22	27.644	4.48	cis 10 hepta decanoic acid	Saturated	17
23	28.735	2.166	oleic acid	unsaturated	18
25	29.94	3.96	(9E, 12E)- octadeca-9,12- decanoic acid	unsaturated	18
26	30.4	12.66	icosanoic acid	Saturated	20
28	31.01	1.06	gamma- linolenic acid	unsaturated	18
29	31.68	0.91	cis 11 eicosanoic acid	unSaturated	20
30	32.26	11.67	octadecatrienoic acid	unsaturated	18

Conclusions

The variation of different growth conditions with the three independent variables such as pH, glucose concentration and KNO_3 concentration has enabled to determine the change in the cell components at the

appropriate combinations with respect to RSM. This performance has made it possible to understand the relative relation between the chosen variables. The significant nature of the lipid at different analytical stress conditions are used to analyse the appropriate stress levels for its maximum yield. The nature of protein content and chlorophyll at various criteria was used for the determination of efficient stress parameters. Therefore a statistically based optimization using CCD method was proved to be useful in optimizing the growth parameters for the higher yield of lipid content in *Chlorella* sp.

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