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Enhancement of Biomass Production of *Chlorella pyrenoidosa* using Response Surface Methodology

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ABSTRACT

The increased biomass production of microalgae is important for the economic production of biofuel. An efficient and economic production biomass requires the optimum values of cultivation parameters such pH value and concentration of KNO_3. In this work, central composite design (CCD) was employed to study the effect of pH and KNO_3 for the biomass production of *Chlorella pyrenoidosa* using Bold Basal Media (BBM). In the first phase, the biomass concentration (g/l) and optical density of *C.pyrenoidosa* was measured for 16 days and formulated a correlation to find biomass concentration from optical density values. Based on our previous experiments, the biomass concentration of *C.pyrenoidosa* was affected by parameters such as concentration of KNO_3 and pH of the BBM used. In the second phase, these parameters are then optimized using response surface methodology with CCD, in which experiments are performed using 13 combinations of these parameters using Minitab-17 software. The optimum values of concentration of KNO_3 and pH were found 0.18 g/l and 8, respectively with an experimental biomass concentration of 1.72 g/l against a predicted value of 1.819 g/l.

Keywords - Central composite design, Biomass concentration, Regression analysis, Bold basal media, pH.

1. INTRODUCTION

The irreversible depletion of fossil fuels and its escalating price, environmental effect due to accumulation of greenhouse gases in the atmosphere requires a clean sustainable alternative source of energy. The biofuels are attractive alternative source of energy which predominantly derived from plant material [1, 2]. The biofuel production from conventional crops such as soybean, palm are limited due to the requirement of more land for cultivation and it makes scarcity of agricultural land for food production [3]. Thus microalgae are considered as a promising feedstock for biofuel production due to their faster growth rate, higher biomass productivity, lipid content. Microalgae can able to synthesize and accumulate substantially higher amount of lipids than terrestrial and their biomass doubling times ranges from 3.5 h to 24 h [4]. The production of biofuel from microalgae involves the following stages such as cultivation, harvesting, biomass processing, lipid extraction and biodiesel production from the lipid extracted [5]. The cultivation of algae with reduced cost is important for minimizing the cost of biodiesel produced. There are number of factors influencing the microalgae growth and are divided as abiotic and biotic factors. The abiotic factors are light, temperature, concentration of KNO_3, O_2, CO_2, pH, salinity and toxic chemicals. The biotic factors are pathogens like bacteria, fungi, viruses, competition by other algae [5]. Among these factors, concentration of KNO_3 in the culture media can regulate the intracellular lipid production and growth [6].

A few literatures are reported about the effect of cultivation parameters such as CO_2, pH, O_2, light, nutrient concentration for the growth of microalgae [6, 7]. But no reports are available for the optimization of KNO_3 concentration and pH for the growth of *C.pyrenoidosa* under room temperature and sunlight. The purpose of this study was to optimize the significant parameters such as concentration of KNO_3 and pH for the biomass concentration of *C.pyrenoidosa* using RSM with CCD. In the first phase, the correlation between biomass concentration and optical density of *C.pyrenoidosa* was obtained from the linear regression analysis of the experimental data. In the second phase, optimization of significant parameters such as

concentration of KNO_3 and pH for biomass concentration of *C.pyrenoidosa* using RSM with CCD was done.

2. MATERIALS AND METHODS

2.1 Collection and stock culture preparation

Microalgae, *Chlorella pyrenoidosa* was procured from National Centre for Industrial Microorganisms (NCIM), Pune, India. The stock culture of *Chlorella pyrenoidosa* was grown in Bold Basal Media (BBM) under room temperature with sunlight. Each litre of BBM contained 0.25 g KNO_3, 0.074 g K_2HPO_4, 0.073 g $MgSO_4 \cdot 7H_2O$, 0.024 g $CaCl_2 \cdot 2H_2O$, 0.025 g NaCl, 0.005 g $FeSO_4$, 0.045 g Na_2EDTA and the pH was adjusted to 7. The preparation of stock culture was done by adding 10% inoculum to the sterilized BBM. Based on the available preliminary experiments and literatures, the concentration of KNO_3 and pH has effect on biomass production of *C.pyrenoidosa*.

2.2 Experimental Design

In order to enhance the growth of *Chlorella pyrenoidosa*, response surface methodology with CCD were employed for experimental design [8]. The total number of experiments for two variables was 13 (2^k+2k+n_o), where k is the number independent variables and n_o is the number of repetition of the experiment at centre point [9]. Totally 13 experiments were formed with 5 replications at the centre values to evaluate the pure error.

The response was measured in terms of biomass concentration (Y). The experimental result was fitted with second order polynomial equation obtained from multiple regression analysis [9,10].

$$Y = b_o + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i=2}^{k} b_{ij} X_i X_j + e_i \quad (1)$$

where Y is the experimental response; X_i and X_j are the independent variables; b_0 is the average of the experimental response; b_i is the estimation of the principal effect of the factor i on the response Y; b_{ii} is the estimation of the second effect of the factor i on the response Y; b_{ij} is the estimation of the interaction effect between i and j on the response Y and e_i represents the error on the response Y. This model was used to estimate the effect of linear, square and interactive of independent variable like concentration of KNO_3 and pH on the dependent variable, biomass concentration i.e, Y. The RSM with CCD and data analysis for checking the quality of fitted polynomial model using value of coefficient of determination, R^2 was done using Minitab-17 software.

Based on our previous studies and literature for the cultivation of *C.pyrenoidosa* under room temperature and sunlight, concentration of KNO_3 and pH was found significant. The parameters such as concentration of KNO_3 (X_1) and pH (X_2) are found significant and are optimized using RSM with CCD. As shown in Table 1, each parameter is coded in five different levels -1.414, -1, 0, +1, +1.414 using Minitab-17 software against the corresponding real values.

Table 1 Levels of factors used

Code	Factor	Levels of factor				
		-1.414	-1	0	+1	+1.414
X_1	KNO_3	0.019	0.05	0.125	0.2	0.231
X_2	pH	5.586	6	7	8	8.414

Two level full factorial design with 13 experiments, 4 experiments as cubical point, 4 as axial points followed by 5 as replications at centre point formed in Minitab-17 software. As shown in Table 2, the various combination of these parameters in the range ($0.019<X_1<0.231$), ($5.586<X_2<8.414$) were used.

2.3 Determination of biomass concentration and optical density

The biomass concentration and optical density of *C.pyrenoidosa* culture was measured on alternate days for total of 16 days. A pre-weighed eppendorf tube of 1.5 ml capacity was used with 1 ml of *C.pyrenoidosa* culture and centrifuged at 6000 rpm for 5 min using a micro-centrifuge. The supernatant obtained was discarded and the pellet remained in the eppendorf tube is dried at 60^{o}C in hot air oven. The drying was continued until the weight of the tube remains constant. One ml of *C.pyrenoidosa* culture is used in glass cuvette of 1 ml capacity to measure the optical density at 680 nm using a UV-visible spectrophotometer.

2.4 Determination of correlation between optical density and biomass concentration

The cultivation of *C.pyrenoidosa* was done in three conical flasks with a working volume of 500 ml. The biomass concentration and corresponding optical density was measured on alternate days from 0th to 16th day of cultivation. Based on this data, regression

analysis was done using Minitab 17 software to arrive a correlation between biomass concentration and optical density.

Table 2 Experimental design using RSM with CCD (X_1: KNO_3, X_2: pH)

Run	X_1	X_2	Biomass concentration (g/l)	
	Real values		Experimental	Predicted
1	0.125	7	1.58	1.584
2	0.125	7	1.58	1.584
3	0.125	5.58	1.07	1.096
4	0.125	7	1.58	1.584
5	0.125	7	1.58	1.584
6	0.05	8	1.47	1.504
7	0.125	8.41	1.76	1.705
8	0.231	7	1.56	1.521
9	0.2	8	1.78	1.852
10	0.019	7	1.52	1.532
11	0.05	6	1.46	1.431
12	0.05	6	1.06	1.431
13	0.125	7	1.58	1.584

3. RESULTS AND DISCUSSION

In the first phase of the work, a correlation between biomass concentration and optical density was formulated. In the second phase, the optimization of significant parameters such as concentration of KNO_3 and pH for the biomass production of *C.pyrenoidosa* was done using RSM with CCD. For validating these experiments, statistical data analysis was done using Minitab-17 software.

3.1 Linear regression analysis

Based on the measured values of optical density and biomass concentration, the regression analysis in Minitab-17 software gave the Eq.(2) as:

$$Y\ (g/l) = 0.0219 + 6.49\ OD_{680\ nm} \quad (2)$$

where Y is the biomass concentration in g/l, $OD_{680\ nm}$ is the optical density measured using UV-visible spectrophotometer at 680 nm. The correlation coefficient, R^2 for the regression analysis is 95.9%, which is nearer to 100 indicates the accuracy of the correlation formulated. The above correlation is used for all optimization study to estimate the biomass concentration of *C.pyrenoidosa* from the measured values of optical density at 680 nm.

3.2 Statistical analysis using RSM

13 experiments were conducted using RSM with different values of KNO_3 and pH. It was found that run number 7 (0.125 g/l KNO_3 and pH of 8.41) showed a maximum biomass concentration of 1.76 g/l. As shown in Table 2, for the same value of KNO_3 concentration of 0.125 g/l, the value of biomass concentration is lesser with different combination of pH.

The result of Analysis of variance (ANOVA) of regression model from the statistical analysis using Minitab-17 software is shown in Table 3. The significance level is checked by using regression model with higher F value and minimum p value ($p<0.05$). Among the linear terms, X_2 is significant with p value less than 0.05. Among the square terms, X_2^2 is significant with p value less than 0.05 and for interactive terms, X_1*X_2 is significant with p value less than 0.05. This result shows the importance of pH for improving the biomass concentration. The estimated values of regression coefficient are used for forming regression model which is a second order polynomial equation as:

$$Y\ (g/l) = -2.386 - 15.34\ KNO_3 + 1.195\ pH - 5.11\ KNO_3*KNO_3 - 0.0912\ pH*pH + 2.367\ KNO_3*pH \quad (3)$$

where Y represents biomass concentration in g/l; X1 is concentration of KNO_3 ($0.019<X_1<0.231$); X_2 is the pH ($5.586<X_2<8.841$). The correlation coefficient, R^2 obtained from the statistical analysis using Minitab-17 software is 97.74% (close to 100) indicates the experimental biomass concentration values are very close to the predicted values obtained from regression model.

Fig. 1 shows a two dimensional contour plot mapped by considering pH in X-axis and concentration of KNO3 in Y-axis. The mutual interaction between the parameters is significant or not is based on the shape of the contour plot. According to Guo *et al*. [11], the circular shaped contour plot shows the interaction between the parameters are not significant whereas the elliptical shaped contour plot shows interactions are significant. Thus, as shown in Fig.1, region in which pH ranges from 7.6 to 8.3 with a maximum KNO3 concentration of 0.075 g/l is elliptical as well as the same trend was

followed with a KNO3 concentration of 0.17 g/l under similar range of pH. Based on our previous study the pH value of 8 eliminates the contamination of C.pyrenoidosa culture with bacteria and fungus.

Table 3 ANOVA of regression coefficients

	Sum of squares	Mean squares	F-value	p-value	Significance
Regression Model	0.549820	0.109964	60.67	0.000	Significant
Linear terms	0.363862	0.181931	100.37	0.000	Significant
X_1	0.000140	0.000140	0.08	0.789	Not significant
X_2	0.363722	0.363722	200.67	0.000	Significant
Square terms	0.059933	0.029966	16.53	0.002	Significant
X_1^2	0.005750	0.005750	3.17	0.118	Not significant
X_2^2	0.057924	0.057924	31.96	0.001	Significant
Interactive terms	0.126025	0.126025	69.53	0.000	Significant
X_1*X_2	0.126025	0.126025	69.53	0.000	Significant

According to Ho *et al.* [7], the biomass concentration of *C.pyrenoidosa* reduced with reduced amount of nitrogen concentration in the cultivation media. Hence, an optimum values of concentration of KNO_3 and pH were found 0.18 g/l and 8, respectively with an experimental biomass concentration of 1.72 g/l.

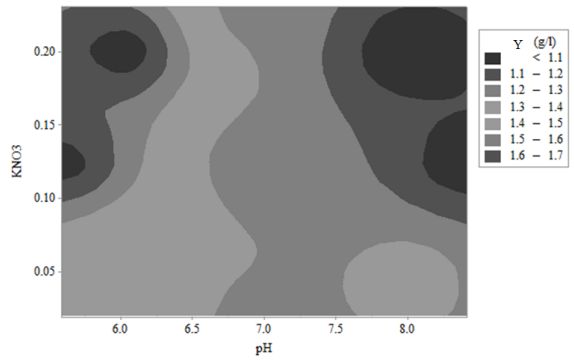

Fig.1 Contour plot for the effect of concentration of KNO_3 and pH

4. CONCLUSIONS

In the first phase of the work, a correlation between the biomass concentration and optical density was formulated for *C.pyrenoidosa*. The parameters such as concentration of KNO_3 and pH was identified significant for the biomass concentration of *C.pyrenoidosa* and are optimized using RSM with CCD. The optimum value of concentration of KNO_3 and pH are 0.18 g/l and 8, respectively with an experimental biomass concentration of 1.72 g/l against a predicted value of 1.819 g/l. Based on regression analysis of experimental data, a second order polynomial equation is formed and found that pH has positive effect on biomass concentration and KNO_3 is insignificant which is also confirmed by the regression analysis. Thus, pH has significant effect on biomass concentration of *C.pyrenoidosa*.

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